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(54) Title: T-BET COMPOSITIONS AND METHODS OF USE THEREOF

(57) Abstract: Isolated nucleic acid molecules encoding T-bet, and isolated T-bet proteins, are provided. The invention further provides antisense nucleic acid molecules, recombinant expression vectors containing a nucleic acid molecule of the invention, host cells into which the expression vectors have been introduced and non-human transgenic animals carrying a T-bet transgene. The invention further provides T-bet fusion proteins and anti-T-bet antibodies. Methods of using the T-bet compositions of the invention are also disclosed, including methods for detecting T-bet activity in a biological sample, methods of modulating T-bet activity in a cell, and methods for identifying agents that modulate the activity of T-bet.

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Background of the Invention

10 Cells of the immune system alter patterns of gene expression in response to extracellular and intracellular signals. A group of polypeptides, designated cytokines or lymphokines, which affect a range of biological activities in several cell types, are among the most important of these signals. While many cell types in the immune system secrete cytokines, the T helper (Th) lymphocyte is the major source of these 15 polypeptides. More than a decade ago it was discovered that Th cells differentiate into two distinct subsets, Th1 and Th2, upon T cell receptor engagement, defined both by their distinct functional abilities and by unique cytokine profiles (Paul and Seder, 1994, Cell 76, 241-251; Mosmann and Coffman, 1989, Annu. Rev. Immunol. 7, 145-173; Mosmann et al., 1986, J. Immunol. 136, 2348-2357; Snapper and Paul, 1987, Science 20 236, 944-947). Th1 cells mediate delayed type hypersensitivity responses and macrophage activation while Th2 cells provide help to B cells and are critical in the allergic response (Mosmann and Coffman, 1989, Annu. Rev. Immunol. 7, 145-173; Paul and Seder, 1994, Cell 76, 241-251; Arthur and Mason, 1986, J. Exp. Med. 163, 774-786; Paliard et al., 1988, J. Immunol. 141, 849-855; Finkelman et al., 1988, J. Immunol. 141, 25 2335-2341). The evidence that Th1 cells directed cell-mediated immunity while Th2 cells contributed to humoral responses fit nicely with the observations that an organism tends to mount either a cell-mediated or humoral response, but not both, in response to pathogens. These functional differences between the Th subsets can be explained most easily by the activities of the cytokines themselves. IFN- γ is the "signature" cytokine of 30 Th1 cells although Th1 cells also produce IL-2, TNF and LT. The corresponding

"signature" cytokine for Th2 cells is IL-4. Th2 cells also secrete IL-5, IL-6, IL-9, IL-10 and IL-13.

Upon encountering antigen, the naive CD4+ T helper precursor (Thp) cell enacts a genetic program that ultimately sends it down a Th1 or Th2 lineage. While it is clear that polarization can be achieved by manipulating the antigen and costimulatory signals i.e. the "strength of signal" received by the Thp (Constant and Bottomly, 1997. Annu. Rev. Immunol. 15, 297-322), the most potent inducers of effector Th cells are undoubtedly the cytokines themselves. IL-4 promotes Th2 differentiation and simultaneously blocks Th1 development, an effect that is mediated via the Stat6 signaling pathway. Thus, mice that lack IL-4 or Stat6, fail to develop Th2 cells (Kopf et al., 1993, Nature 362, 245-248; Kuhn et al., 1991, Science 254, 707-710; Kaplan et al., 1996, Immunity 4, 313-319; Shimoda et al., 1996, Nature 380, 630-633; Takeda et al., 1996, Nature 380, 627-630). In contrast, IL-12, IL-18 and IFN- γ are the cytokines critical for the development of Th1 cells (Hsieh et al., 1993, Science 260, 547-549; Okamura et al., 1995, nature 378, 88-91; Gu et al., 1997, Science 275, 206-209; Meraz et al., 1996, Cell 84, 431-442; Magram et al., 1996, Immunity 4, 471-481). IFN- γ , acting via the Stat1 pathway (Meraz et al., 1996, Cell 84, 431-442), and IL-12, acting via the Stat-4 signaling pathway (Jacobson et al., 1995, J. Exp. Med. 181, 1755-1762) together promote the differentiation of Th1 cells and block commitment to the Th2 lineage (Szabo et al., 1995, Immunity 2, 665-675; Szabo et al., 1997, J. Exp. Med. 185: 817-824). Mice deficient in IL-12 or Stat4 do not have Th1 cells (Magram et al., 1996, Immunity 4, 471-481; Takeda et al., 1996, Nature 380, 627-630; Shimoda et al., 1996, Nature 380, 630-633). Another important Th1-inducing cytokine is IL-18, whose receptor is related to the IL-1 receptor family (Cerretti et al., 1992, Science 256, 97-100). Mice lacking IL-18 have defective in vivo Th1 responses (Takeda et al., 1998, Immunity 8, 383-390) and both IL-12 and IL-18 regulate IFN- γ expression (Barbulescu et al., 1998, Eur. J. Immunol. 27, 1098-1107; Robinson et al., 1997, Immunity 7, 571-581; Ahn et al., 1997, J. Immunol. 159, 2125-2131). The cytokines themselves, then, form a positive and negative feedback system that drives Th polarization (Powrie and Coffman, 1993, Immunol. Today 14, 270-274; Scott, 1991, J. Immunol. 147, 3149;

Maggi et al., 1992, *J. Immunol.* 148, 2142; Parronchi et al., 1992, *J. Immunol.* 149, 2977; Fargeas et al., 1992, *Eur. J. Immunol.* 149, 2977; Manetti et al., 1993, *J. Exp. Med.* 177, 1199; Trinchieri, 1993, *Immunol. Today* 14, 335-338; Macatonia et al., 1993, *Immunol. Rev.* 5, 1119; Seder et al., 1993, *Proc. Natl. Acad. Sci. USA* 90, 10188-10192; Wu et al., 1993, *J. Immunol.* 151, 1938; Hsieh et al., 1993, *Science* 260, 547-549) (reviewed in (Seder and Paul, 1994, *In Annual Review of Immunology*, Vol. 12, 635-673; Paul and Seder, 1994, *Cell* 76, 241-251; O'Garra, 1998, *Immunity* 8, 275-283).

Over the last few years, significant progress has been made in identifying the transcription factors that control the transition of a Thp to a Th2 cell as evidenced by the 10 capacity of such factors to drive IL-4 production reviewed in (Glimcher and Singh, 1999 *Cell* 96, 13-23; Szabo et al., 1997, *Current Opinions in Immunology* 9, 776-781). The provision of three distinct proteins, the c-Maf proto-oncogene, the transcription factor Nuclear Factor of Activated T cells (NFAT), and a novel nuclear antigen, NFAT-Interacting Protein 45 kD (NIP45), have been shown to confer on a non-T cell the ability 15 to produce endogenous IL-4 (Hodge et al., 1996, *Science* 274, 1903-1905; Ho et al., 1998, *J. Exp. Med.* 188:1859-1866). These factors and others such as GATA-3 (Zheng and Flavell, 1997, *Cell* 89, 587-596) and Stat6 clearly can drive the production of IL-4, and therefore the development of Th2 cells, both *in vitro* and *in vivo*.

In contrast, little is known about the molecular basis of Th1 differentiation. For 20 example, the only known transcription factors whose absence results in a failure to generate Th1 cells are Stat4 (Thierfelder et al., 1996, *Nature* 382, 171-174; Kaplan et al., 1996, *Nature* 382, 174-177) and IRF-1 (Lohhoff et al., 1997, *Immunity* 6:681-689; Taki et al., 1997, *Immunity* 6:673-679), neither of which is Th1-specific. The Ets family member ERM which is induced by IL-12 in a Stat4-dependent manner has recently been 25 reported to be Th1-specific but it does not affect the production of Th1 cytokines (Ouyang et al., 1999, *Proc. Natl. Acad. Sci.* 96:3888). The absence of Th1 cells in Stat4 deficient mice is secondary to the failure of IL-12 to drive the Th1 program while the lack of Th1 cells in IRF-1 deficient mice is likely due to its direct effect in controlling transcription of the IL-12 gene (Lohhoff et al., 1997, *Immunity* 6: 681-689; Taki et al., 30 1997, *Immunity* 6:673-679). However, some of the signaling pathways upstream of such putative Th1-specific regulatory factors are beginning to be elucidated. The p38 kinase

is one such signaling molecule as demonstrated by the ability of constitutively activated MAP kinase kinase 6 (MKK6) to boost IFN- γ production. Conversely, overexpression of a dominant negative p38 MAP kinase or targeted disruption of Jnk2 or Jnk1 reduces Th1 responses (Rincón et al., 1998, EMBO J. 17, 2817-2829; Yang et al., 1998, Immunity 9, 5 575-585; Dong et al., 1998, Science 282, 2092-2095). The JNK signaling pathway might affect Th development by a direct effect on the transcription of the IFN- γ gene, but this has not been shown. For example, the ATF-2 and AP-1 transcription factors are both substrates of JNK kinases and these factors as well as NF κ B and Stat4 proteins are known to bind to sites in the IFN- γ promoter (Zhang et al., 1998, Immunol. 161, 6105-10 6112; Ye et al., 1996, Mol. Cell. Biol. 16:4744; Barbulescu et al., 1997, Eur. J. Immunol. 27, 1098-1107; Sica et al., 1997, J. Biol. Chem. 272, 30412-30420). The production of IFN- γ is, however, normal in mice lacking ATF-2. Because cytokines are critical in the development of Th1 and Th2 cells and, thereby, in determining whether an immune response will be primarily cellular or humoral, compositions and methods for 15 modulating the production of Th1 and/or Th2 cytokines would be of tremendous benefit in modulating the immune response.

Summary of the Invention

This invention is based, at least in part, on the discovery of novel compositions 20 which act to promote the Th1 phenotype in naïve T helper precursor cells (Thp), both by initiating Th1 cell genetic programs and by repressing the opposing programs in Th2 cells. In particular, this invention provides isolated nucleic acid molecules encoding T-bet and isolated T-bet protein. T-bet (T_box expressed in T cells) is a new member of the T box family of transcription factors whose founding member is the *brachyury* gene. T- 25 bet is constitutively expressed selectively in thymocytes and Th1 cells. T-bet is the first Th1 specific transcription factor that can transactivate the interferon-gamma gene, induce interferon-gamma production in retrovirally transduced primary T cells and redirect polarized Th2 cells into the Th1 pathway. The invention also provides methods of using these novel T-bet compositions.

One aspect of the invention pertains to an isolated nucleic acid molecule comprising a nucleotide sequence encoding T-bet. In a preferred embodiment, the nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 1 or 3. In other embodiments, the nucleic acid molecule comprises at least 700 contiguous 5 nucleotides of SEQ ID NO:1 or at least 500 contiguous nucleotides of SEQ ID NO:3. In a preferred embodiment, the nucleic acid molecule has at least 70 % nucleotide identity, with at least 700 contiguous nucleotides of SEQ ID NO:1 or at least 70% identity with at least 500 contiguous nucleotides of SEQ ID NO:3.

The isolated nucleic acid molecules of the invention encoding T-bet can be 10 incorporated into a vector, such as an expression vector, and this vector can be introduced into a host cell. The invention also provides a method for producing a T-bet protein by culturing a host cell of the invention (carrying a T-bet expression vector) in a suitable medium until a T-bet protein is produced. The method can further involve isolating the T-bet protein from the medium or the host cell.

15 Another aspect of the invention pertains to an isolated T-bet protein. Preferably, the T-bet protein comprises the amino acid sequence of SEQ ID NO: 2 or 4. In other embodiments, the protein has at least 60 % amino acid identity, at least 70% amino acid identity, more preferably 80 % amino identity, at, and even more preferably 90 % amino acid identity with the amino acid sequence shown in SEQ ID NO:1 or 3.

20 Fusion proteins, comprising a T-bet protein operatively linked to a polypeptide other than T-bet, are also encompassed by the invention, as well as antibodies that specifically bind a T-bet protein. The antibodies can be, for example, polyclonal antibodies or monoclonal antibodies. In one embodiment, the antibodies are coupled to a detectable substance.

25 Another aspect of the invention pertains to a nonhuman transgenic animal that contains cells carrying a transgene encoding a T-bet protein.

Yet another aspect of the invention pertains to a method for detecting the presence of T-bet in a biological sample. The method involves contacting the biological sample with an agent capable of detecting an indicator of T-bet activity such that the 30 presence of T-bet is detected in the biological sample. The invention also provides a method for modulating T-bet activity in a cell comprising, involving contacting the cell

with an agent that modulates T-bet activity such that T-bet activity in the cell is modulated.

Still another aspect of the invention pertains to methods for identifying a compound that modulates the activity of a T-bet protein. These methods generally

5 involve:

providing an indicator composition that comprises a T-bet protein;

contacting the indicator composition with a test compound; and

determining the effect of the test compound on the activity of the T-bet protein in the indicator composition to thereby identify a compound that modulates the activity of

10 a T-bet protein. In a preferred embodiment, the indicator composition comprises a T-bet protein and a DNA molecule to which the T-bet protein binds and the effect of the test compound on the activity of the T-bet protein is determined by evaluating the binding of the T-bet protein to the DNA molecule in the presence and absence of the test compound. In another preferred embodiment, the indicator composition is a cell

15 comprising a T-bet protein and a reporter gene responsive to the T-bet protein and the effect of the test compound on the activity of the T-bet protein is determined by evaluating the expression of the reporter gene in the presence and absence of the test compound. In yet another embodiment, the method further involves the step of determining the effect of the test compound on an immune response to thereby identify a

20 compound that modulates an immune response.

Brief Description of the Drawings

Figure 1A shows a nucleotide sequence alignment of murine and human T-bet. The alignment was prepared using the ALIGN program. Figure 1B shows an amino acid sequence alignment of murine and human T-bet prepared using the Lipman Pearson protein alignment program. The T-box sequence is shown in bold. Tyrosine phosphorylation sites are underlined. The nuclear localization site is marked with arrows.

30 Figure 2 A and B show that T-bet binds to and transactivates consensus T-box sites with functionally important domains that map to both 5' and 3' regions.

Figure 3A shows that T-bet is preferentially expressed in double negative thymocytes. Panel B shows that in a survey of Th clones, T-bet expression is restricted to Th1 cells. Panel C shows western blot analysis of T-bet. Panel D shows FACS analysis of T-bet expression..

Figure 4 A and B show that T-bet expression correlates with IFN- γ induction in NK and B cells.

10 Figure 5 shows that T-bet transactivates the IFN- γ gene in Th cells.

Figure 6 shows that retroviral gene transduction of T-bet reduces increases IFN-gamma production and represses IL-2 production.

15 Figure 7 shows that T-bet activates IFN- γ and represses IL-2 production in primary T cells.

Figure 8 shows that T-bet induces IFN-gamma and inhibits IL-4 production in developing Th2 cells.

20 Figure 9 shows that T-bet redirects polarized Th2 cells into the Th1 pathway. Th skewing was carried out as above and retroviral infections were performed on day 9 of culture

25 Figure 10 shows that T-bet redirects polarized Tc2 cells into the Tc1 pathway. CD8+ T cells were purified by MoFlo and cultured under Th2 skewing conditions as above and retroviral transductions performed on day 8 of culture.

Figure 11 shows that T-bet is tyrosine phosphorylated.

30 Figure 12 shows the activity of a T-bet dominant negative mutant.

Figure 13 shows that mutations of the T-box element of the IL-2 promoter reduce IL-2 promoter activity.

5 Detailed Description of the Invention

This invention pertains to T-bet compositions, such as isolated nucleic acid molecules encoding T-bet and isolated T-bet proteins, as well as methods of use therefore.

So that the invention may be more readily understood, certain terms are first 10 defined.

As used herein, the term "T-bet molecules" includes T-bet nucleic acid molecules that share structural features with the nucleic acid molecules shown in SEQ ID Nos: 1 and 3 and T-bet proteins that share the distinguishing structural and functional features of the T-bet proteins shown in SEQ ID Nos 2 and 4. The T-bet proteins are 15 members of the T-box family of proteins and share some amino acid sequence homology to Brachury, Tbx1-6, T-brain-1 (Tbr-1). T-box proteins comprise a T box domain which binds to DNA at a T box binding site. Further structural and functional features of T-bet proteins are provided below.

As used herein, the term "nucleic acid molecule" is intended to include DNA 20 molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA). The nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

As used herein, an "isolated nucleic acid molecule" refers to a nucleic acid molecule that is free of gene sequences which naturally flank the nucleic acid in the 25 genomic DNA of the organism from which the nucleic acid is derived (*i.e.*, genetic sequences that are located adjacent to the gene for the isolated nucleic molecule in the genomic DNA of the organism from which the nucleic acid is derived). For example, in various embodiments, an isolated T-bet nucleic acid molecule typically contains less than about 10 kb of nucleotide sequences which naturally flank the nucleic acid 30 molecule in genomic DNA of the cell from which the nucleic acid is derived, and more preferably contains less than about 5, kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of

naturally flanking nucleotide sequences. An "isolated" T-bet nucleic acid molecule may, however, be linked to other nucleotide sequences that do not normally flank the T-bet sequences in genomic DNA (e.g., the T-bet nucleotide sequences may be linked to vector sequences). In certain preferred embodiments, an "isolated" nucleic acid

5 molecule, such as a cDNA molecule, also may be free of other cellular material. However, it is not necessary for the T-bet nucleic acid molecule to be free of other cellular material to be considered "isolated" (e.g., a T-bet DNA molecule separated from other mammalian DNA and inserted into a bacterial cell would still be considered to be "isolated").

10 As used herein, the term "hybridizes under high stringency conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences having substantial homology (e.g., typically greater than 70% homology) to each other remain stably hybridized to each other. A preferred, non-limiting example of high stringency conditions are hybridization in a hybridization buffer that contains 6X

15 sodium chloride/ sodium citrate (SSC) at a temperature of about 45°C for several hours to overnight, followed by one or more washes in a washing buffer containing 0.2 X SSC, 0.1% SDS at a temperature of about 50-65°C.

The term "percent (%) identity" as used in the context of nucleotide and amino acid sequences (e.g., when one amino acid sequence is said to be X% identical to

20 another amino acid sequence) refers to the percentage of identical residues shared between the two sequences, when optimally aligned. To determine the percent identity of two nucleotide or amino acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps may be introduced in one sequence for optimal alignment with the other sequence). The residues at corresponding positions are then

25 compared and when a position in one sequence is occupied by the same residue as the corresponding position in the other sequence, then the molecules are identical at that position. The percent identity between two sequences, therefore, is a function of the number of identical positions shared by two sequences (i.e., % identity = # of identical positions/total # of positions x 100).

Computer algorithms known in the art can be used to optimally align and compare two nucleotide or amino acid sequences to define the percent identity between the two sequences. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul

5 1990 Proc. Natl. Acad. Sci. USA 87:2264-68, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-77. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. To obtain gapped alignments for comparison purposes. Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Research 25(17):3389-10 3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>. For example, the nucleotide sequences of the invention were blasted using the default Blastn matrix 1-3 with gap penalties set at: existance 5 and extension 2. The amino acid sequences of the invention were blasted using the 15 default settings: the Blosum62 matrix with gap penalties set at existance 11 and extension 1.

Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part 20 of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. If multiple programs are used to compare sequences, the program that provides optimal alignment (i.e., the highest percent identity between the two sequences) is used for comparison purposes.

25 As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (e.g., encodes a natural protein).

As used herein, an "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, e.g., 30 complementary to the coding strand of a double-stranded cDNA molecule,

complementary to an mRNA sequence or complementary to the coding strand of a gene. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid.

As used herein, the term "coding region" refers to regions of a nucleotide sequence comprising codons which are translated into amino acid residues, whereas the 5 term "noncoding region" refers to regions of a nucleotide sequence that are not translated into amino acids (e.g., 5' and 3' untranslated regions).

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional 10 DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the 15 genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of 20 plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

25 As used herein, the term "host cell" is intended to refer to a cell into which a nucleic acid of the invention, such as a recombinant expression vector of the invention, has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It should be understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because 30 certain modifications may occur in succeeding generations due to either mutation or

environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

As used herein, a "transgenic animal" refers to a non-human animal, preferably a mammal, more preferably a mouse, in which one or more of the cells of the animal 5 includes a "transgene". The term "transgene" refers to exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, for example directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal.

As used herein, a "homologous recombinant animal" refers to a type of 10 transgenic non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

As used herein, an "isolated protein" refers to a protein that is substantially free 15 of other proteins, cellular material and culture medium when isolated from cells or produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized.

As used herein, the term "antibody" is intended to include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, 20 molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, such as Fab and F(ab')₂ fragments. The terms "monoclonal antibodies" and "monoclonal antibody composition", as used herein, refer to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen, whereas the term "polyclonal 25 antibodies" and "polyclonal antibody composition" refer to a population of antibody molecules that contain multiple species of antigen binding sites capable of interacting with a particular antigen. A monoclonal antibody compositions thus typically display a single binding affinity for a particular antigen with which it immunoreacts.

There is a known and definite correspondence between the amino acid sequence 30 of a particular protein and the nucleotide sequences that can code for the protein, as defined by the genetic code (shown below). Likewise, there is a known and definite

correspondence between the nucleotide sequence of a particular nucleic acid molecule and the amino acid sequence encoded by that nucleic acid molecule, as defined by the genetic code.

5 GENETIC CODE

	Alanine (Ala, A)	GCA, GCC, GCG, GCT
	Arginine (Arg, R)	AGA, ACG, CGA, CGC, CGG, CGT
	Asparagine (Asn, N)	AAC, AAT
	Aspartic acid (Asp, D)	GAC, GAT
10	Cysteine (Cys, C)	TGC, TGT
	Glutamic acid (Glu, E)	GAA, GAG
	Glutamine (Gln, Q)	CAA, CAG
	Glycine (Gly, G)	GGA, GCC, GGG, GGT
	Histidine (His, H)	CAC, CAT
15	Isoleucine (Ile, I)	ATA, ATC, ATT
	Leucine (Leu, L)	CTA, CTC, CTG, CTT, TTA, TTG
	Lysine (Lys, K)	AAA, AAG
	Methionine (Met, M)	ATG
	Phenylalanine (Phe, F)	TTC, TTT
20	Proline (Pro, P)	CCA, CCC, CCG, CCT
	Serine (Ser, S)	AGC, AGT, TCA, TCC, TCG, TCT
	Threonine (Thr, T)	ACA, ACC, ACG, ACT
	Tryptophan (Trp, W)	TGG
	Tyrosine (Tyr, Y)	TAC, TAT
25	Valine (Val, V)	GTA, GTC, GTG, GTT
	Termination signal (end)	TAA, TAG, TGA

An important and well known feature of the genetic code is its redundancy, whereby, for most of the amino acids used to make proteins, more than one coding nucleotide triplet 30 may be employed (illustrated above). Therefore, a number of different nucleotide sequences may code for a given amino acid sequence. Such nucleotide sequences are considered functionally equivalent since they result in the production of the same amino acid sequence in all organisms (although certain organisms may translate some sequences more efficiently than they do others). Moreover, occasionally, a methylated 35 variant of a purine or pyrimidine may be found in a given nucleotide sequence. Such

methylations do not affect the coding relationship between the trinucleotide codon and the corresponding amino acid.

In view of the foregoing, the nucleotide sequence of a DNA or RNA molecule coding for a T-bet protein of the invention (or any portion thereof) can be used to derive 5 the T-bet amino acid sequence, using the genetic code to translate the DNA or RNA molecule into an amino acid sequence. Likewise, for any T-bet-amino acid sequence, corresponding nucleotide sequences that can encode the T-bet protein can be deduced from the genetic code (which, because of its redundancy, will produce multiple nucleic acid sequences for any given amino acid sequence). Thus, description and/or disclosure 10 herein of a T-bet nucleotide sequence should be considered to also include description and/or disclosure of the amino acid sequence encoded by the nucleotide sequence. Similarly, description and/or disclosure of a T-bet amino acid sequence herein should be considered to also include description and/or disclosure of all possible nucleotide sequences that can encode the amino acid sequence.

15 *Brachyury* or *T* is the founding member of a family of transcription factors that share a 200 amino acid DNA-binding domain called the T-box (reviewed in (Smith, 1997; Papaioannou, 1997; Meissner, 1997)). The *Brachyury* (Greek for 'short tail') mutation was first described in 1927 in heterozygous mutant animals who had a short, slightly kinked tail (Herrmann et al., 1990). The amino-terminal half (amino acids 1- 20 229) of the *Brachyury* T-box protein contains a conserved domain known as the T box which has been shown to exhibit sequence-specific DNA-binding activity (Kispert, A. & Herrmann, B. G. 1993. *EMBO J.* 12:3211; Papapetrou, C., et al. 1997. *FEBS Lett.* 409:201; Kispert, A., et al. 1995. *EMBO J.* 14:4763). The C-terminal half contains two pairs of transactivation and repression domains. The similarity of sequence between the 25 T box region in orthologous species can be as high as 99% and is around 40-70% between non-orthologous genes. The T-box domain has recently been co-crystallized with DNA and demonstrates a novel sequence-specific DNA recognition architecture in which the protein contacts DNA in both the major and minor grooves (Müller, C. W. & Herrmann, B. G. 1997. *Nature* 389, 884.

A yeast one hybrid approach was used to identify Th-1 specific transcription factors. Yeast cells were made to express an IL-2 promoter-reporter gene construct and were transformed with a cDNA library made from an anti-CD3 activated Th1 cell clone. Inspection of the IL-2 promoter reveals an excellent T-box binding site at -240 to -220 5 just 5' of the NF κ B site. As described in the appended examples, T-bet was isolated in a yeast one hybrid screening assay based on its ability to bind to the IL-2 promoter.

The nucleotide sequence encoding murine T-bet is shown in SEQ ID NO:3. Murine T-bet is a 530 amino acid protein with a 190 amino acid T-box domain located at residues 136-326. The amino acid sequence of murine T-bet is shown in SEQ ID 10 NO:4. After the murine T-bet sequence was cloned as described herein, it was possible to compile the sequence of the human ortholog of T-bet from nucleic acid fragments which were not previously known to encode any known protein. The nucleotide sequence of human T-bet is shown in SEQ ID NO:1. Human T-bet is a 535 amino acid protein with a 190 amino acid T-box domain located at residues 138-327. The human T- 15 bet gene maps to chromosome 17. The nucleotide and amino acid sequences of two members (human and mouse) of the T-bet family of proteins are shown in Figure 1 and SEQ ID Nos: 1-4.

The T-bet proteins of the invention have homology to T-box proteins. There are now eight T-box genes in the mouse not including *Brachury*. These include Tbx1-6, T- 20 brain-1 (*Tbr-1*) and now, T-bet, each with a distinct and usually complex expression pattern. T-brain-1 expression, for example is largely restricted to distinct domains within the cerebral cortex (Bulfone, A., et al. 1995. *Neuron* 15, 63. T-bet is most similar in sequence to *Tbr-1*. Outside of the T-box, the T-bet proteins of the invention bear no similarity to other T-box proteins.

25 T-bet is T-box protein expressed only in T cells and is most similar in sequence to *Tbr-1*. Other species also express *Brachury*-like genes. Such vertebrate species include Xenopus, zebrafish, chick and humans (Rao, 1994; Horb and Thomsen, 1997; Conlon et al., 1996; Ryan et al., 1996; Schulte-Merker et al., 1994; Edwards et al., 1996; Morrison et al., 1996; Law et al., 1995; Cambell et al., 1998) as well as more distant 30 species such as amphioxus, ascidians, echinoderms, *Caenorhabditis elegans*, *Drosophila*

and other insects (Holland et al., 1995). These genes are conserved both in sequence and in expression pattern.

T-bet is unique in that it is the only T-box protein to be tyrosine phosphorylated. There are two consensus tyrosine phosphorylation sites at aa 328-336 and 526-534 of

5 human T-bet and 327-335 and 521-529 of murine T-bet. A nuclear localization sequence is also present at amino acids 498-501 of human T-bet and 493-496 of murine T-bet. Mapping experiments locate two transactivation domains, one 5' and one 3' of the T-box domain. The data shown herein demonstrate that T-bet binds to a consensus T-box site (defined by target site selection *in vitro* as 5'-

10 GGGAAATTCACACCTAGGTGTGAAATTCCC-3') and to a T-box site in the IL-2 promoter. T-bet is expressed only in the thymus and in the peripheral lymphoid system. In the periphery, T-bet is expressed only in Th1 cells where it is induced both in response to TcR stimulation and to IL-12. In the thymus levels of T-bet are highest in DN and Rag2-/-thymocytes.

15 These data demonstrate that the selective expression of T-bet, a novel T-box family member, accounts for tissue-specific IFN- γ expression. T-bet is expressed only in Th1 and not in Th2 cells and is induced in the former upon transmission of signals through the T cell receptor. The expression of T-bet correlates with IFN- γ expression in Th1 cells, NK cells and B cells, and T-bet is a potent transactivator of the IFN- γ gene.

20 Most convincing, retroviral mediated transduction of Thp, Th1 and polarized Th2 and Tc2 cells with T-bet results in an impressive induction of IFN- γ expression. This is accompanied by repression of both IL-2 and IL-4 production. Thus, the function of T-bet extends beyond the simple control of IFN- γ gene transcription. T-bet converts both polarized effector Th2 cells and polarized Tc2 cells into the opposing Th1 and Tc1

25 subsets, respectively. Taken together, these data demonstrate that T-bet is responsible for the genetic program that initiates Th1 lineage development from naïve Thp cells and acts both by initiating Th1 genetic programs and by repressing the opposing programs in Th2 cells.

Various aspects of the invention are described in further detail in the following subsections:

I. Isolated Nucleic Acid Molecules

5 One aspect of the invention pertains to isolated nucleic acid molecules that encode T-bet. In a preferred embodiment, the nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:3. In another embodiment, a nucleic acid molecule of the invention comprises at least about 700 contiguous nucleotides of SEQ ID NO:1 or at least about 500 contiguous nucleotides of

10 SEQ ID NO:3. In a preferred embodiment, a nucleic acid molecule of the invention comprises at least about 800, at least about 1000, at least about 1200, at least about 1400 or at least about 1600 contiguous nucleotides of SEQ ID NO:1. In another preferred embodiment, a nucleic acid molecule of the invention comprises at least about 600, at least about 800, at least about 1000, at least about 1200, or at least about 1400

15 contiguous nucleotides of SEQ ID NO:3.

In other embodiments, the nucleic acid molecule has at least 70 % identity, more preferably 80% identity, and even more preferably 90% identity with a nucleic acid molecule comprising: at least about 700, at least about 800, at least about 1000, at least about 1200, at least about 1400 or at least about 1600 contiguous nucleotides of SEQ ID NO:1. In other embodiments, the nucleic acid molecule has at least 70 % identity, more preferably 80% identity, and even more preferably 90% nucleotide identity with a nucleic acid molecule comprising: at least about 600, at least about 800, at least about 1000, at least about 1200, or at least about 1400 contiguous nucleotides of SEQ ID NO:3.

25 Nucleic acid molecules that differ from SEQ ID NO: 1 or 3 due to degeneracy of the genetic code, and thus encode the same T-bet protein as that encoded by SEQ ID NO: 1 and 3, are encompassed by the invention. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention has a nucleotide sequence encoding a protein having an amino acid sequence shown in SEQ ID NO: 2 or SEQ ID NO:4.

In addition, nucleic acid molecules encoding T-bet proteins can be isolated from other sources using standard molecular biology techniques and the sequence information provided herein. For example, a T-bet DNA can be isolated from a human genomic DNA library using all or portion of SEQ ID NO:1 or 3 as a hybridization probe and

5 standard hybridization techniques (e.g., as described in Sambrook, J., *et al. Molecular Cloning: A Laboratory Manual*. 2nd. ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). Moreover, a nucleic acid molecule encompassing all or a portion of a T-bet gene can be isolated by the polymerase chain reaction using oligonucleotide primers designed based upon the sequence of SEQ ID NO: 1 or 3. For example, mRNA

10 can be isolated from cells (e.g., by the guanidinium-thiocyanate extraction procedure of Chirgwin *et al.* (1979) *Biochemistry* 18: 5294-5299) and cDNA can be prepared using reverse transcriptase (e.g., Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD; or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for PCR

15 amplification can be designed based upon the nucleotide sequence shown in SEQ ID NO: 1 or 3. A nucleic acid of the invention can be amplified using cDNA or, alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis.

20 Furthermore, oligonucleotides corresponding to a T-bet nucleotide sequence can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

In addition to the T-bet nucleotide sequence shown in SEQ ID NO: 1 and 3, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to minor changes in the nucleotide or amino acid sequences of T-bet may exist

25 within a population. Such genetic polymorphism in the T-bet gene may exist among individuals within a population due to natural allelic variation. Such natural allelic variations can typically result in 1-2 % variance in the nucleotide sequence of the a gene. Any and all such nucleotide variations and resulting amino acid polymorphisms in T-bet that are the result of natural allelic variation and that do not alter the functional activity

30 of T-bet are intended to be within the scope of the invention.

Nucleic acid molecules corresponding to natural allelic variants of the T-bet DNAs of the invention can be isolated based on their homology to the T-bet nucleic acid molecules disclosed herein using the human DNA, or a portion thereof, as a hybridization probe according to standard hybridization techniques under high

5 stringency hybridization conditions. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention hybridizes under high stringency conditions to a second nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 1 or 3. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under high stringency conditions to the sequence of SEQ ID NO: of SEQ ID NO:1 or 3. In one

10 embodiment, such a nucleic acid molecule is at least about 700, 800, 900, 1000, 1200, 1300, 1400, 1500, or 1600 nucleotides in length. In another embodiment, such a nucleic acid molecule and comprises at least about 700, 800, 900, 1000, 1200, 1300, 1400, 1500, or 1600 contiguous nucleotides of SEQ ID NO: 1 or at least about 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, or 1500 contiguous nucleotides of SEQ ID NO: 3.

15 Preferably, an isolated nucleic acid molecule corresponds to a naturally-occurring allelic variant of a T-bet nucleic acid molecule.

In addition to naturally-occurring allelic variants of the T-bet sequence that may exist in the population, the skilled artisan will further appreciate that minor changes may be introduced by mutation into the nucleotide sequence of SEQ ID NO: 1 or 3, thereby

20 leading to changes in the amino acid sequence of the encoded protein, without altering the functional activity of the T-bet protein. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues may be made in the sequence of SEQ ID NO: 1 or 3. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of T-bet (e.g., the sequence of SEQ ID

25 NO: 1 or 3) without altering the functional activity of T-bet, such as its ability to interact with DNA or its ability to enhance transcription from an IFN-gamma promoter, whereas an "essential" amino acid residue is required for functional activity.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding T-bet proteins that contain changes in amino acid residues that are not essential

30 for T-bet activity. Such T-bet proteins differ in amino acid sequence from SEQ ID NO: 2 or 4 yet retain T-bet activity. An isolated nucleic acid molecule encoding a non-

natural variant of a T-bet protein can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of SEQ ID NO: 1 or 3 such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into SEQ ID NO: 1 or 3 by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis.

5 Preferably, conservative amino acid substitutions are made at one or more non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art,

10 including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g.,

15 tyrosine, phenylalanine, tryptophan, histidine). Thus, a nonessential amino acid residue in T-bet is preferably replaced with another amino acid residue from the same side chain family.

Alternatively, in another embodiment, mutations can be introduced randomly along all or part of the T-bet coding sequence, such as by saturation mutagenesis, and 20 the resultant mutants can be screened for their ability to bind to DNA and/or activate transcription, to identify mutants that retain functional activity. Following mutagenesis, the encoded T-bet mutant protein can be expressed recombinantly in a host cell and the functional activity of the mutant protein can be determined using assays available in the art for assessing T-bet activity (e.g., by measuring the ability of the protein to bind to a 25 T-box binding element present in DNA or by measuring the ability of the protein to modulate a Th1 or Th2 phenotype in a T cell).

Another aspect of the invention pertains to isolated nucleic acid molecules that are antisense to the coding strand of a T-bet mRNA or gene. An antisense nucleic acid of the invention can be complementary to an entire T-bet coding strand, or to only a 30 portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a coding region of the coding strand of a nucleotide sequence encoding T-bet that is

unique to the T-bet family of proteins or which is unique to a T-bet sequence from a particular species. In another embodiment, the antisense nucleic acid molecule is antisense to a noncoding region of the coding strand of a nucleotide sequence encoding T-bet that is unique to T-bet family of proteins or which is unique to a T-bet sequence
5 from a particular species. In preferred embodiments, an antisense molecule of the invention comprises at least about 700 contiguous nucleotides of the noncoding strand of SEQ ID NO: 1, more preferably at least 800, 1000, 1200, 1400, or 1600 contiguous nucleotides of the noncoding strand of SEQ ID NO: 1 or at least about 500 contiguous nucleotides of the noncoding strand of SEQ ID NO: 3, more preferably at least 600,
10 800, 1000, 1200, or 1400 contiguous nucleotides of the noncoding strand of SEQ ID NO: 3.

Given the coding strand sequences encoding T-bet disclosed herein (e.g., SEQ ID NOs: 1 and 3, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule may be
15 complementary to the entire coding region of T-bet mRNA, or alternatively can be an oligonucleotide which is antisense to only a portion of the coding or noncoding region of T-bet mRNA. For example, the antisense oligonucleotide may be complementary to the region surrounding the translation start site of T-bet mRNA. An antisense oligonucleotide can be, for example, about 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in
20 length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical
25 stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense
30 orientation to a target nucleic acid of interest, described further in the following subsection).

In another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozyomes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. A ribozyme having specificity for a T-bet-encoding nucleic acid 5 can be designed based upon the nucleotide sequence of a T-bet gene disclosed herein. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the base sequence of the active site is complementary to the base sequence to be cleaved in a T-bet-encoding mRNA. See for example Cech *et al.* U.S. Patent No. 4,987,071; and Cech *et al.* U.S. Patent No. 5,116,742. Alternatively, T-bet mRNA can be used to select 10 a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See for example Bartel, D. and Szostak, J.W. (1993) *Science* 261: 1411-1418.

Yet another aspect of the invention pertains to isolated nucleic acid molecules encoding T-bet fusion proteins. Such nucleic acid molecules, comprising at least a first nucleotide sequence encoding a T-bet protein, polypeptide or peptide operatively linked 15 to a second nucleotide sequence encoding a non-T-bet protein, polypeptide or peptide, can be prepared by standard recombinant DNA techniques. T-bet fusion proteins are described in further detail below in subsection III.

II. Recombinant Expression Vectors and Host Cells

20 Another aspect of the invention pertains to vectors, preferably recombinant expression vectors, containing a nucleic acid encoding T-bet (or a portion thereof). The expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on 25 the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the 30 vector is introduced into the host cell). The term "regulatory sequence" includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation

signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185. Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the 5 nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion 10 proteins or peptides, encoded by nucleic acids as described herein (e.g., T-bet proteins, mutant forms of T-bet proteins, T-bet fusion proteins and the like).

The recombinant expression vectors of the invention can be designed for expression of T-bet protein in prokaryotic or eukaryotic cells. For example, T-bet can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression 15 vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector may be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

20 Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors can serve one or more purposes: 1) to increase expression of recombinant 25 protein; 2) to increase the solubility of the recombinant protein; 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification; 4) to provide an epitope tag to aid in detection and/or purification of the protein; and/or 5) to provide a marker to aid in detection of the protein (e.g., a color marker using β -galactosidase fusions). Often, in fusion expression vectors, a proteolytic cleavage site is 30 introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification

of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc.; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which 5 fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. Recombinant proteins also can be expressed in eukaryotic cells as fusion proteins for the same purposes discussed above.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.* (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gnl0-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gnl). This viral 10 polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 gnl gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the 20 recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, (1992) *Nuc. Acids Res.* 20:2111-2118). 25 Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the T-bet expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYEpSec1 (Baldari. *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-30 943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA).

Alternatively, T-bet can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith *et al.*, (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pMex-Neol, pCDM8 (Seed, B., (1987) *Nature* 329:840) and pMPT2PC (Kaufman *et al.* (1987), *EMBO J.* 6:187-195). When used in mammalian cells, 10 the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type 15 (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) 20 *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Patent No. 25 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α -fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

Moreover, inducible regulatory systems for use in mammalian cells are known in 30 the art, for example systems in which gene expression is regulated by heavy metal ions (see e.g., Mayo *et al.* (1982) *Cell* 29:99-108; Brinster *et al.* (1982) *Nature* 296:39-42;

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Searle *et al.* (1985) *Mol. Cell. Biol.* 5:1480-1489), heat shock (see *e.g.*, Nouer *et al.* (1991) in *Heat Shock Response*, e.d. Nouer, L. , CRC, Boca Raton , FL, pp167-220), hormones (see *e.g.*, Lee *et al.* (1981) *Nature* 294:228-232; Hynes *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78:2038-2042; Klock *et al.* (1987) *Nature* 329:734-736; Israel & 5 Kaufman (1989) *Nucl. Acids Res.* 17:2589-2604; and PCT Publication No. WO 93/23431), FK506-related molecules (see *e.g.*, PCT Publication No. WO 94/18317) or tetracyclines (Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Gossen, M. *et al.* (1995) *Science* 268:1766-1769; PCT Publication No. WO 94/29442; and PCT Publication No. WO 96/01313). Accordingly, in another 10 embodiment, the invention provides a recombinant expression vector in which T-bet DNA is operatively linked to an inducible eukaryotic promoter, thereby allowing for inducible expression of T-bet protein in eukaryotic cells.

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense 15 orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to T-bet mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for 20 instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell 25 type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. *et al.*, Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to recombinant host cells into which a vector, preferably a recombinant expression vector, of the invention has been introduced. 30 A host cell may be any prokaryotic or eukaryotic cell. For example, T-bet protein may be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells

(such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art. Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of 5 art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and 10 other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is 15 generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker may be introduced into a host cell on the same vector as that encoding T-bet or may be introduced on a separate vector. Cells stably transfected with the introduced nucleic 20 acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (i.e., express) T-bet protein. Accordingly, the invention further provides methods for producing T-bet protein using the host cells of the 25 invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding T-bet has been introduced) in a suitable medium until T-bet is produced. In another embodiment, the method further comprises isolating T-bet from the medium or the host cell. In its native form the T-bet protein is an intracellular protein and, accordingly, recombinant T-bet 30 protein can be expressed intracellularly in a recombinant host cell and then isolated from the host cell, e.g., by lysing the host cell and recovering the recombinant T-bet protein

from the lysate. Alternatively, recombinant T-bet protein can be prepared as a extracellular protein by operatively linking a heterologous signal sequence to the amino-terminus of the protein such that the protein is secreted from the host cells. In this case, recombinant T-bet protein can be recovered from the culture medium in which the cells 5 are cultured.

Certain host cells of the invention can also be used to produce nonhuman transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which T-bet-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic 10 animals in which exogenous T-bet sequences have been introduced into their genome or homologous recombinant animals in which endogenous T-bet sequences have been altered. Such animals are useful for studying the function and/or activity of T-bet and for identifying and/or evaluating modulators of T-bet activity. Accordingly, another aspect of the invention pertains to nonhuman transgenic animals which contain cells 15 carrying a transgene encoding a T-bet protein or a portion of a T-bet protein. In a subembodiment, of the transgenic animals of the invention, the transgene alters an endogenous gene encoding an endogenous T-bet protein (e.g., homologous recombinant animals in which the endogenous T-bet gene has been functionally disrupted or "knocked out", or the nucleotide sequence of the endogenous T-bet gene has been 20 mutated or the transcriptional regulatory region of the endogenous T-bet gene has been altered).

A transgenic animal of the invention can be created by introducing T-bet-encoding nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, and allowing the oocyte to develop in a pseudopregnant female foster 25 animal. The T-bet nucleotide sequence of SEQ ID NO: 1 or 3 can be introduced as a transgene into the genome of a non-human animal. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to the T-bet transgene to direct expression of T-bet protein to particular cells. 30 Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are

described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder *et al.*, U.S. Patent No. 4,873,191 by Wagner *et al.* and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A 5 transgenic founder animal can be identified based upon the presence of the T-bet transgene in its genome and/or expression of T-bet mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding T-bet can further be bred to other transgenic animals carrying other transgenes.

10 To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a T-bet gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the endogenous T-bet gene. In one embodiment, a homologous recombination vector is designed such that, upon homologous recombination, the endogenous T-bet gene is functionally disrupted (*i.e.*, no 15 longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous T-bet gene replaced by the T-bet gene. In the homologous recombination vector, the altered portion of the T-bet gene is flanked at its 5' and 3' ends by additional nucleic acid of the T-bet gene to allow for homologous recombination to 20 occur between the exogenous T-bet gene carried by the vector and an endogenous T-bet gene in an embryonic stem cell. The additional flanking T-bet nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see *e.g.* Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a 25 description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced T-bet gene has homologously recombined with the endogenous T-bet gene are selected (see *e.g.*, Li, E. *et al.* (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, 30 A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be

implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing 5 homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.*

In addition to the foregoing, the skilled artisan will appreciate that other 10 approaches known in the art for homologous recombination can be applied to the instant invention. Enzyme-assisted site-specific integration systems are known in the art and can be applied to integrate a DNA molecule at a predetermined location in a second target DNA molecule. Examples of such enzyme-assisted integration systems include the Cre recombinase-lox target system (e.g., as described in Baubonis, W. and Sauer, B. 15 (1993) *Nucl. Acids Res.* 21:2025-2029; and Fukushige, S. and Sauer, B. (1992) *Proc. Natl. Acad. Sci. USA* 89:7905-7909) and the FLP recombinase-FRT target system (e.g., as described in Dang, D.T. and Perrimon, N. (1992) *Dev. Genet.* 13:367-375; and Fiering, S. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:8469-8473). Tetracycline-regulated inducible homologous recombination systems, such as described in PCT 20 Publication No. WO 94/29442 and PCT Publication No. WO 96/01313, also can be used.

In another embodiment, transgenic animals can be made in which T-bet is expressed in all T cells, e.g., using the CD4 enhancer (Zheng, W-P. & Flavell, R. A. 1997. *Cell* 89, 587). Recent work suggests the CD2 enhancer can also be used. In fact, it 25 is more powerful in achieving high level expression in T cells, expression is not variegated and transgene expression is copy number-dependent (Zhumabekov, T., *et al.* 1995. *J. Immunol. Meth.* 185, 133; Sharp, L. L., *et al.* 1997. *Immunity* 7, 609). Mice with high level expression of T-bet RNA (using the human growth hormone intron as a probe to distinguish transgene driven T-bet RNA from endogenous T-bet) can be identified by 30 screening adequate numbers of founders.

In another approach, a dominant repressor transgenic can be created. For example, a dominant-repressor T-bet can be made by using the proximal lck enhancer (Alberola-Ila, J., et al. 1996 *J. Exp. Med.* 184, 9) driving a fusion of T-bet and engrailed can be made (Taylor, D., 1996. *Genes Dev.* 10, 2732; Li, J., Thurm, H., et al. 1997. 5 *Proc. Natl. Acad. Sci. USA* 94, 10885). This construct specifically represses T-bet transactivation of a multimerized T-bet reporter and does not affect NFAT-dependent reporter transactivation.

Alternatively, null mutations can be generated by targeted mutagenesis in ES cells (Ranger, A. M., et al. 1998. *Nature* 392, 186; Hodge, M. R., et al. 1996. *Immunity* 10, 41; 144; Grusby, M. J., et al. 1991. *Science* 253, 1417; Reimold, A. M., et al. 1996. *Nature* 379: 262; Kaplan, M. H., 1996. *Immunity* :313; Kaplan, M. H., et al. 1996. *Nature* 382, 174; Smiley, S. T., et al. 1997. *Science* 275, 977). For example using techniques which are known in the art, a genomic T-bet clone can be isolated from a genomic library, the intron-exon organization delineated, and a targeting construct in the 15 cre-lox vector (see discussion below) created which should delete the first exon and 450 bp of upstream promoter sequence. This construct can be electroporated into an ES cell line, and double drug resistant (e.g., neomycin, gancyclovir) clones identified by Southern blot analysis. Clones bearing homologous recombinant events in the T-bet locus can then be identified and injected into blastocysts obtained from day 3.5 BALB/c 20 pregnant mice. Chimeric mice can then be produced and mated to wildtype BALB/c mice to generate germline transmission of the disrupted T-bet gene.

In another embodiment, implantation into RAG2-deficient blastocysts (Chen, J., et al. 1993. *Proc. Natl. Acad. Sci. USA* 90, 4528) or the cre-lox inducible deletion approach can be used to develop mice that are lacking T-bet only in the immune system. 25 For example, the targeting construct can be made in the cre-lox vector. The blastocyst complementation system has been used to study NFATc, an embryonic lethal phenotype (Ranger, A. M., et al. 1998. *Immunity* 8:125). This approach requires disrupting the T-bet gene on both chromosomes in ES cells, which can be accomplished, e.g., by using a mutant neomycin gene and raising the concentration of G418 in the ES cultures, as 30 described (Chen, J., 1993. *Proc. Natl. Acad. Sci. USA* 90:4528) or by flanking the neo gene with cre-lox sites. To disrupt the second allele, the neomycin gene can be deleted

by transfecting the ES clone with the cre recombinase, and then the ES clone can be retransfected with the same targeting construct to select clones with T-bet deletions on both alleles. A third transfection with cre-recombinase yields the desired doubly-deficient ES cells. Such doubly targeted ES cells are then implanted into RAG2

5 blastocysts and the lymphoid organs of the chimeric mice thus generated will be entirely colonized by the transferred ES cells. This allows assessment of the effect of the absence of T-bet on cells of the lymphoid system without affecting other organ systems where the absence of T-bet might cause lethality.

The conditional ablation approach employing the cre-lox system can also be

10 used. Briefly, a targeting construct is generated in which lox recombination sequences are placed in intronic regions flanking the exons to be deleted. This construct is then transfected into ES cells and mutant mice are generated as above. The resulting mutant mice are then mated to mice transgenic for the cre recombinase driven by an inducible promoter. When cre is expressed, it induces recombination between the introduced lox

15 sites in the T-bet gene, thus effectively disrupting gene function. The key feature of this approach is that gene disruption can be induced in the adult animal at will by activating the cre recombinase.

A tissue-specific promoter can be used to avoid abnormalities in organs outside the immune system. The cre-expressing transgene may be driven by an inducible

20 promoter. Several inducible systems are now being used in cre-lox recombination strategies, the most common being the tetracycline and ecdysone systems. A tissue-specific inducible promoter can be used if there is embryonic lethality in the T-bet null mouse.

An alternative approach is to generate a transgenic mouse harboring a regulated

25 T-bet gene (for example using the tetracycline off promoter; e.g., St-Onge, et al. 1996. *Nuc. Acid Res.* 24, 3875-3877) and then breed this transgenic to the T-bet deficient mouse. This approach permits creation of mice with normal T-bet function; tetracycline can be administered to adult animals to induce disruption of T-bet function in peripheral T cells, and then the effect of T-bet deficiency can be examined over time. Repeated

30 cycles of provision and then removal of drug (tetracycline) permits turning the T-bet gene on and off at will.

III. Isolated T-bet Proteins and Anti-T-bet Antibodies

Another aspect of the invention pertains to isolated T-bet proteins. Preferably, the T-bet protein comprises the amino acid sequence encoded by SEQ ID NO:1 or 3. In another preferred embodiment, the protein comprises the amino acid sequence of SEQ 5 ID NO: 2 or 4. In other embodiments, the protein has at least 60 % amino acid identity, more preferably 70% amino acid identity, more preferably 80%, and even more preferably, 90% or 95% amino acid identity with the amino acid sequence shown in SEQ ID NO: 2 or 4.

In other embodiments, the invention provides isolated portions of the T-bet 10 protein. For example, the invention further encompasses an amino-terminal portion of T-bet that includes a T-box domain. In various embodiments, this amino terminal portion encompasses at least amino acids 138-327 of human T-bet or at least amino acids 137-326 of mouse T-bet. Another isolated portion of T-bet provided by the invention is a portion encompassing a tyrosine phosphorylation site. This portion 15 encompasses at least amino acids 324-366 and/or 523-534 of human T-bet or amino acids 323-335 or 518-529 of murine T-bet. Yet another isolated portion of T-bet provided herein is a portion encompassing a nuclear localization sequence shown in amino acids 498-501 of human T-bet or 493-496 of murine T-bet.

T-bet proteins of the invention are preferably produced by recombinant DNA 20 techniques. For example, a nucleic acid molecule encoding the protein is cloned into an expression vector (as described above), the expression vector is introduced into a host cell (as described above) and the T-bet protein is expressed in the host cell. The T-bet protein can then be isolated from the cells by an appropriate purification scheme using standard protein purification techniques. Alternative to recombinant expression, a T-bet 25 polypeptide can be synthesized chemically using standard peptide synthesis techniques. Moreover, native T-bet protein can be isolated from cells (e.g., from T cells), for example by immunoprecipitation using an anti-T-bet antibody.

The invention also provides T-bet fusion proteins. As used herein, a T-bet "fusion protein" comprises a T-bet polypeptide operatively linked to a polypeptide other 30 than T-bet. A "T-bet polypeptide" refers to a polypeptide having an amino acid sequence corresponding to T-bet protein, or a peptide fragment thereof which is unique

to T-bet protein whereas a "polypeptide other than T-bet" refers to a polypeptide having an amino acid sequence corresponding to another protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the T-bet polypeptide and the other polypeptide are fused in-frame to each other. The other polypeptide may be fused to the 5 N-terminus or C-terminus of the T-bet polypeptide. For example, in one embodiment, the fusion protein is a GST-T-bet fusion protein in which the T-bet sequences are fused to the C-terminus of the GST sequences. In another embodiment, the fusion protein is a T-bet-HA fusion protein in which the T-bet nucleotide sequence is inserted in a vector such as pCEP4-HA vector (Herrschner, R.F. *et al.* (1995) *Genes Dev.* 9:3067-3082) such 10 that the T-bet sequences are fused in frame to an influenza hemagglutinin epitope tag. Such fusion proteins can facilitate the purification of recombinant T-bet.

Preferably, a T-bet fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional 15 techniques, for example employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR 20 amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that 25 already encode a fusion moiety (e.g., a GST polypeptide or an HA epitope tag). A T-bet-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the T-bet protein.

An isolated T-bet protein, or fragment thereof, can be used as an immunogen to generate antibodies that bind specifically to T-bet using standard techniques for 30 polyclonal and monoclonal antibody preparation. The T-bet protein can be used to generate antibodies. For example, polyclonal antisera, can be produced in rabbits using

full-length recombinant bacterially produced T-bet as the immunogen. This same immunogen can be used to produce mAb by immunizing mice and removing spleen cells from the immunized mice. Spleen cells from mice mounting an immune response to T-bet can be fused to myeloma cells, e.g., SP2/O-Ag14 myeloma. As described in the 5 appended examples, this methods were used to make polyclonal and monoclonal antibodies which bind to T-bet.

Alternatively, an antigenic peptide fragment of T-bet can be used as the immunogen. An antigenic peptide fragment of T-bet typically comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO: 2 or 4 and 10 encompasses an epitope of T-bet such that an antibody raised against the peptide forms a specific immune complex with T-bet. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues. Preferred epitopes encompassed by the antigenic peptide are regions of T-bet 15 that are located on the surface of the protein, e.g., hydrophilic regions, and that are unique to T-bet. In one embodiment such epitopes can be specific for T-bet proteins from one species, such as mouse or human (i.e., an antigenic peptide that spans a region of T-bet that is not conserved across species is used as immunogen; such non conserved residues can be determined using an alignment such as that provided herein). A standard 20 hydrophobicity analysis of the T-bet protein can be performed to identify hydrophilic regions.

A T-bet immunogen typically is used to prepare antibodies by immunizing a suitable subject, (e.g., rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for examples, recombinantly 25 expressed T-bet protein or a chemically synthesized T-bet peptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic T-bet preparation induces a polyclonal anti-T-bet antibody response.

Accordingly, another aspect of the invention pertains to anti-T-bet antibodies. 30 Polyclonal anti-T-bet antibodies can be prepared as described above by immunizing a suitable subject with a T-bet immunogen. The anti-T-bet antibody titer in the

immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized T-bet. If desired, the antibody molecules directed against T-bet can be isolated from the mammal (e.g., from the blood) and further purified by well known techniques, such as protein A

5 chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the anti-T-bet antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256:495-497) (see also, Brown *et al.* (1981) *J. Immunol* 127:539-10 46; Brown *et al.* (1980) *J Biol Chem* 255:4980-83; Yeh *et al.* (1976) *PNAS* 73:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985). *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal 15 antibody hybridomas is well known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L. Gefter *et al.* (1977) *Somatic Cell Genet.*, 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal 20 immunized with a T-bet immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds specifically to T-bet.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-T-bet 25 monoclonal antibody (see, e.g., G. Galfré *et al.* (1977) *Nature* 266:55052; Gefter *et al.* *Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth, *Monoclonal Antibodies*, cited *supra*). Moreover, the ordinary skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same 30 mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of

the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques, 5 *e.g.*, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Md. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively 10 fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind T-bet, *e.g.*, using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a 15 monoclonal anti-T-bet antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (*e.g.*, an antibody phage display library) with T-bet to thereby isolate immunoglobulin library members that bind T-bet. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and 20 the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* International Publication No. WO 92/18619; Dower *et al.* International Publication No. WO 91/17271; Winter *et al.* International Publication 25 WO 92/20791; Markland *et al.* International Publication No. WO 92/15679; Breitling *et al.* International Publication WO 93/01288; McCafferty *et al.* International Publication No. WO 92/01047; Garrard *et al.* International Publication No. WO 92/09690; Ladner *et al.* International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) 30 *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.*

(1992) *PNAS* 89:3576-3580; Garrad *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; Barbas *et al.* (1991) *PNAS* 88:7978-7982; and McCafferty *et al.* *Nature* (1990) 348:552-554.

Additionally, recombinant anti-T-bet antibodies, such as chimeric and

5 humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.* International Patent Publication PCT/US86/02269; Akira, *et al.*

10 European Patent Application 184,187; Taniguchi, M.. European Patent Application 171,496; Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT Application WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *PNAS* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *PNAS* 84:214-218; Nishimura *et al.* (1987) *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl Cancer Inst.* 80:1553-1559); Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *BioTechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeven *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.*

15 20 141:4053-4060.

An anti-T-bet antibody (e.g., monoclonal antibody) can be used to isolate T-bet by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-T-bet antibody can facilitate the purification of natural T-bet from cells and of recombinantly produced T-bet expressed in host cells. Moreover, an anti-T-bet antibody

25 can be used to detect T-bet protein (e.g., in a cellular lysate or cell supernatant). Detection may be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Accordingly, in one embodiment, an anti-T-bet antibody of the invention is labeled with a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials

30 and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of

suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and 5 examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S or ^3H .

Yet another aspect of the invention pertains to anti-T-bet antibodies that are obtainable by a process comprising:

- (a) immunizing an animal with an immunogenic T-bet protein, or an immunogenic portion thereof unique to T-bet protein; and
- 10 (b) isolating from the animal antibodies that specifically bind to a T-bet protein.

Methods for immunization and recovery of the specific anti-T-bet antibodies are described further above.

IV. Pharmaceutical Compositions

15 T-bet modulators of the invention (e.g., T-bet inhibitory or stimulatory agents, including T-bet nucleic acid molecules, proteins, antibodies, or compounds identified as modulators of T-bet activity) can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the modulatory agent and a pharmaceutically acceptable carrier. As used herein the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof 20 in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

25 A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. For example, solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents;

antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, 5 such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the 10 extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy 15 syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the 20 maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include 25 isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of 30 ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle

which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient

5 from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared

10 using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as

15 microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

20 In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhdydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid.

25 Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled

30 in the art, for example, as described in U.S. Patent No. 4,522,811.

V. Methods of the Invention

Another aspect of the invention pertains to methods of using the various T-bet compositions of the invention. For example, the invention provides a method for detecting the presence of T-bet activity in a biological sample. The method involves 5 contacting the biological sample with an agent capable of detecting T-bet activity, such as T-bet protein or T-bet mRNA, such that the presence of T-bet activity is detected in the biological sample.

A preferred agent for detecting T-bet mRNA is a labeled nucleic acid probe capable of specifically hybridizing to T-bet mRNA. The nucleic acid probe can be, for 10 example, the T-bet DNA of SEQ ID NO: 1 or 3, such as an oligonucleotide of at least about 500, 600, 800, 900, 1000, 1200, 1400, or 1600 nucleotides in length and which specifically hybridizes under stringent conditions to T-bet mRNA.

A preferred agent for detecting T-bet protein is a labeled antibody capable of binding to T-bet protein. Antibodies can be polyclonal, or more preferably, monoclonal. 15 An intact antibody, or a fragment thereof (e.g., Fab or F(ab')₂) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include 20 detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids. For example, techniques for detection of T-bet mRNA include Northern hybridizations and *in situ* hybridizations. Techniques for detection of T-bet 25 protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence.

Such assays are useful in detecting syndromes characterized by developmental defects. For example, mutations in the human T-box genes *TBX5* and *TBX3* (orthologs of mouse *Tbx5* and *Tbx3*) are responsible for the autosomal dominant genetic diseases 30 Holt-Oram syndrome and ulnar-mammary syndrome respectively (Bamshad, M., et al. 1997. *Nature Genetics* 16: 311; Basson, C. T., et al. 1997. *Nature Genetics* 15:30; Li,

Q. Y., et al. 1997. *Nature Genetics* 15: 21; Spranger, S., et al. 1997. *J. Med. Genet.* 3:978). These syndromes are characterized by developmental defects and might have been predicted by the patterns of expression of Tbx5 and Tbx3 respectively. Holt-Oram syndrome affects the heart and upper limbs while ulnar-mammary syndrome affects 5 limb, apocrine gland, tooth and genital development. Both syndromes are characterized by developmental defects and might have been predicted by the patterns of expression of Tbx5 and Tbx3 respectively. The mutations in these patients involve only one allele of the T-box gene- thus it has been postulated that haploinsufficiency of Tbx3 and Tbx 5 cause these two diseases. Recently it has been demonstrated that provision of Tbx4 and 10 Tbx5 to developing chick embryos controls limb bud identity (Rodriguez-Esteban et al., 1999; Takeuchi et al.. 1999). These discoveries emphasize the critical importance of this family in vertebrate development.

In addition, the existence of T gene homologs in many species provides strong 15 evidence for its function as a transcription factor that regulates a set of as yet unknown target genes involved in mesoderm development. The recent prominence of the T-box family arises from its clear importance in diverse developmental processes, exemplified most dramatically by the T-box mutations in human disease. The generation of mature T cells from thymocyte stem cells and of differentiated Th cells from naive precursors can also be viewed as tightly regulated developmental processes. This discovery that T-bet is 20 responsible for the development of the Th1 lineage demonstrates an important role for this newest T-box family member in the lymphoid system.

The invention further provides methods for identifying compounds that modulate the activity of a T-bet protein. For example, the invention provides a method for identifying a compound that modulates the activity of a T-bet protein, comprising 25 providing an indicator composition that comprises a T-bet protein; contacting the indicator composition with a test compound; and determining the effect of the test compound on the activity of the T-bet protein in the indicator composition to thereby identify a compound that modulates the activity of a T-bet protein.

Specific embodiments of the screening methods of the invention exploit the ability of T-bet proteins to bind to DNA (e.g., the ability to bind to an IL-2 or IFN-gamma promoter) and/or to regulate gene expression (e.g., regulate expression of a Th1-associated cytokine gene, e.g., by repressing the IL-2 gene, transactivate the IFN- γ gene) 5 and/or to redirect polarized Th2 cells into the Th1 pathway.

In a preferred embodiment of the screening assays of the invention, the indicator composition comprises an indicator cell, wherein said indicator cell comprises: (i) the a T-bet protein and (ii) a reporter gene responsive to the T-bet protein. Preferably, the indicator cell contains:

- 10 i) a recombinant expression vector encoding the T-bet; and
- ii) a vector comprising regulatory sequences of a Th1-associated cytokine gene operatively linked a reporter gene; and said method comprising:
 - a) contacting the indicator cell with a test compound;
 - b) determining the level of expression of the reporter gene in the indicator cell in
- 15 15 the presence of the test compound; and
- c) comparing the level of expression of the reporter gene in the indicator cell in the presence of the test compound with the level of expression of the reporter gene in the indicator cell in the absence of the test compound to thereby identify a compound that modulates the activity of T-bet.
- 20 In another preferred embodiment, the indicator composition comprises a preparation of: (i) a T-bet protein and (ii) a DNA molecule to which the T-bet binds, and said method comprising:
 - a) contacting the indicator composition with a test compound;
 - b) determining the degree of interaction of the T-bet protein and the DNA
- 25 25 molecule in the presence of the test compound; and
- c) comparing the degree of interaction of the T-bet and the DNA molecule in the presence of the test compound with the degree of interaction of the T-bet protein and the DNA molecule in the absence of the test compound to thereby identify a compound that modulates the activity of T-bet.
- 30 30 Preferably, the DNA molecule to which T-bet binds comprises a T-box binding sequence.

In another preferred embodiment, the method identifies proteins that interact with T-bet. In this embodiment,

the indicator composition is an indicator cell, which indicator cell comprises:

- 5 i) a reporter gene operably linked to a transcriptional regulatory sequence; and
- ii) a first chimeric gene which encodes a first fusion protein, said first fusion protein including T-bet;

the test compound comprises a library of second chimeric genes, which library encodes second fusion proteins;

- 10 expression of the reporter gene being sensitive to interactions between the first fusion protein, the second fusion protein and the transcriptional regulatory sequence; and
- wherein the effect of the test compound on T-bet in the indicator composition is determined by determining the level of expression of the reporter gene in the indicator cell to thereby identify a test compound comprising a protein that interacts with T-bet.

- 15 In a preferred embodiment, the library of second chimeric genes is prepared from cDNA library from Th2 cells.

In a preferred embodiment of the screening assays of the invention, once a test compound is identified as modulating the activity of T-bet, the effect of the test compound on an immune response is then tested. Accordingly, the screening methods 20 of the invention can further comprise determining the effect of the compound on an immune response to thereby identify a compound that modulates an immune response. In one embodiment, the effect of the compound on an immune response is determined by determining the effect of the compound on expression of a Th1-associated cytokine gene, such as an interferon-gamma gene. As used herein, the term "Th1-associated 25 cytokine" is intended to refer to a cytokine that is produced preferentially or exclusively by Th1 cells rather than by Th2 cells. Examples of Th1-associated cytokines include IFN-gamma, IL-2, TNF, and lymphotoxin (LT). In another embodiment, the effect of the compound of interest on an immune response is determined by determining the effect of the compound on development of T helper type 1 (Th1) or T helper type 2 (Th2) cells.

Recombinant expression vectors that can be used for expression of T-bet in the indicator cell are known in the art (see discussions above). In one embodiment, within the expression vector the T-bet-coding sequences are operatively linked to regulatory sequences that allow for constitutive expression of T-bet in the indicator cell (e.g., viral

5 regulatory sequences, such as a cytomegalovirus promoter/enhancer, can be used). Use of a recombinant expression vector that allows for constitutive expression of T-bet in the indicator cell is preferred for identification of compounds that enhance or inhibit the activity of T-bet. In an alternative embodiment, within the expression vector the T-bet-coding sequences are operatively linked to regulatory sequences of the endogenous T-

10 bet gene (*i.e.*, the promoter regulatory region derived from the endogenous T-bet gene). Use of a recombinant expression vector in which T-bet expression is controlled by the endogenous regulatory sequences is preferred for identification of compounds that enhance or inhibit the transcriptional expression of T-bet.

In methods in which a Th1-associated cytokine gene is utilized (e.g., as a reporter gene), preferably, the Th1-associated cytokine is interferon-gamma or IL-2. For example, the IL-2 promoter reveals a T-box binding site at -240 to -220 just 5' of the NF κ B site. As described in the appended examples, T-bet was isolated in a yeast one hybrid screening assay based on its ability to bind to the IL-2 promoter. Accordingly, in one embodiment, a method of the invention utilizes a reporter gene construct containing 20 this region of the proximal IL-2 promoter, most preferably nucleotides -240 to -220 of the IL-2 promoter.

A variety of reporter genes are known in the art and are suitable for use in the screening assays of the invention. Examples of suitable reporter genes include those which encode chloramphenicol acetyltransferase, beta-galactosidase, alkaline 25 phosphatase or luciferase. Standard methods for measuring the activity of these gene products are known in the art.

A variety of cell types are suitable for use as an indicator cell in the screening assay. Preferably a cell line is used which does not normally express T-bet, such as a B cell or a Th2 cell clone. Nonlymphoid cell lines can also be used as indicator cells, such 30 as the HepG2 hepatoma cell line. Yeast cells also can be used as indicator cells.

In one embodiment, the level of expression of the reporter gene in the indicator cell in the presence of the test compound is higher than the level of expression of the reporter gene in the indicator cell in the absence of the test compound and the test compound is identified as a compound that stimulates the expression or activity of T-bet.

5 In another embodiment, the level of expression of the reporter gene in the indicator cell in the presence of the test compound is lower than the level of expression of the reporter gene in the indicator cell in the absence of the test compound and the test compound is identified as a compound that inhibits the expression or activity of T-bet.

Alternative to the use of a reporter gene construct, compounds that modulate the expression or activity of T-bet can be identified by using other "read-outs." For example, an indicator cell can be transfected with a T-bet expression vector, incubated in the presence and in the absence of a test compound, and Th1-associated cytokine production can be assessed by detecting cytokine mRNA (e.g., interferon-gamma mRNA) in the indicator cell or cytokine secretion (i.e., interferon-gamma) into the culture supernatant. Standard methods for detecting cytokine mRNA, such as reverse transcription-polymerase chain reaction (RT-PCR) are known in the art. Standard methods for detecting cytokine protein in culture supernatants, such as enzyme linked immunosorbent assays (ELISA) are also known in the art.

As described above, the invention provides a screening assay for identifying 20 proteins (e.g., proteins in Th1 cells) that interact with T-bet.

In one embodiment, such assays can be designed based on the two-hybrid assay system (also referred to as an interaction trap assay) known in the art (see e.g., Field U.S. Patent No. 5,283,173; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; and 25 Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696). The two-hybrid assay is generally used for identifying proteins that interact with a particular target protein. The assay employs gene fusions to identify proteins capable of interacting to reconstitute a functional transcriptional activator. The transcriptional activator consists of a DNA-binding domain and a transcriptional activation domain, wherein both domains are required to 30 activate transcription of genes downstream from a target sequence (such as an upstream activator sequence (UAS) for GAL4). DNA sequences encoding a target "bait" protein

are fused to either of these domains and a library of DNA sequences is fused to the other domain. "Fish" fusion proteins (generated from the fusion library) capable of binding to the target-fusion protein (e.g., a target GAL4-fusion "bait") will generally bring the two domains (DNA-binding domain and transcriptional activation domain) into close enough proximity to activate the transcription of a reporter gene inserted downstream from the target sequence. Thus, the "fish" proteins can be identified by their ability to reconstitute a functional transcriptional activator (e.g., a functional GAL4 transactivator).

This general two-hybrid system can be applied to the identification of proteins in cells (e.g., Th1 cells) that interact with T-bet by construction of a target T-bet fusion protein (e.g., a T-bet/GAL4 binding domain fusion as the "bait") and a cDNA library of "fish" fusion proteins (e.g., a cDNA/GAL4 activation domain library), wherein the cDNA library is prepared from mRNA of a cell type of interest (e.g., Th1 cells), and introducing these constructs into a host cell that also contains a reporter gene construct linked to a regulatory sequence responsive to T-bet (e.g., an IL-2 promoter sequence, for example, as discussed above). Preferably, the transactivation domain(s) of T-bet will be (which have been mapped to both the 5' and 3' ends) will be deleted in the "bait" construct. In a preferred embodiment, the bait construct will include the T-box domain. In one embodiment, at least one site of tyrosine phosphorylation will also be included.

Dominant negative T-bet proteins can also be used to screen for interactors to further localize sites of T-bet which are required for interaction. cDNAs encoding proteins that interact with T-bet can be identified based upon transactivation of the reporter gene construct.

Alternatively, a "single-hybrid" assay, such as that described in Sieweke, M.H. *et al.* (1996) *Cell* 85:49-60, can be used to identify proteins that interact with T-bet. This assay is a modification of the two-hybrid system discussed above. In this system, the "bait" is a transcription factor from which the transactivation domain has been removed (e.g., T-bet from which a transactivation domain has been removed) and the "fish" is a non-fusion cDNA library (e.g., a cDNA library prepared from Th1 cells). These constructs are introduced into host cells (e.g., yeast cells) that also contains a reporter gene construct linked to a regulatory sequence responsive to T-bet (e.g., comprising a T-

box binding a region, such as a region of the IL-2 promoter responsive to T-bet). cDNAs encoding proteins that interact with T-bet can be identified based upon transactivation of the reporter gene construct.

In another embodiment, representational difference analysis (RDA) and

5 microchip DNA array analysis to isolate T-bet target genes. For example, differential display or subtraction methods coupled with PCR (RDA; see e.g., Hubank, M. & Schatz, D. G. 1994. *Nuc. Acid Res.* **22**, 5640-5648; Chang, Y., et al. 1994. *Science* **266**, 1865; von Stein, O. D., et al. 1997. *Nuc. Acid Res.* **25**, 2598; Lisitsyn, N. & Wigler, M. 1993. *Science* **259**, 946) employing subtracted or unsubtracted probes or, most recently, DNA

10 microchip array hybridization (Welford et al. 1998. *Nucl. Acids. Res.* **15**:3059) can be used. In performing such assays, a variety of cells can be used, e.g., normal cells, cells engineered to express T-bet, or cells from mice lacking T-bet or overexpressing T-bet (e.g., from a transgenic non-human animal) can be used.

As described above, the invention provides a screening assay for identifying

15 compounds that modulate the interaction of T-bet and a T-box binding region (e.g., an IL-2 gene regulatory region). Assays are known in the art that detect the interaction of a DNA binding protein with a target DNA sequence (e.g., electrophoretic mobility shift assays, DNase I footprinting assays and the like). By performing such assays in the presence and absence of test compounds, these assays can be used to identify

20 compounds that modulate (e.g., inhibit or enhance) the interaction of the DNA binding protein with its target DNA sequence.

In one embodiment, the amount of binding of T-bet to the DNA fragment in the presence of the test compound is greater than the amount of binding of T-bet to the DNA fragment in the absence of the test compound, in which case the test compound is

25 identified as a compound that enhances binding of T-bet. In another embodiment, the amount of binding of T-bet to the DNA fragment in the presence of the test compound is less than the amount of binding of T-bet to the DNA fragment in the absence of the test compound, in which case the test compound is identified as a compound that inhibits binding of T-bet.

Yet another aspect of the invention pertains to methods of modulating T-bet activity in a cell. The modulatory methods of the invention involve contacting the cell with an agent that modulates T-bet activity such that T-bet activity in the cell is modulated. The agent may act by modulating the activity of T-bet protein in the cell or 5 by modulating transcription of the T-bet gene or translation of the T-bet mRNA. As used herein, the term "modulating" is intended to include inhibiting or decreasing T-bet activity and stimulating or increasing T-bet activity. Accordingly, in one embodiment, the agent inhibits T-bet activity. In another embodiment, the agent stimulates T-bet activity.

10 In yet another aspect, the invention provides a method for modulating amount of T helper-type 2 and/or T helper-type 1 cytokines by a cell. The method involves contacting a cell with an agent that modulates the activity of T-bet. For example, agents which stimulate T-bet activity upregulate the Th1 cytokine IFN-gamma, while these same agents down regulate the Th2 cytokine IL-4.

15 In a further aspect, the invention provides a method for modulating the pattern of cytokines which is produced by a cell. The method involves contacting a cell with an agent that modulates the activity of T-bet. For example, agents which stimulate T-bet activity can induce IFN-gamma production in a cell which does not normally produce IFN-gamma and can repress IL-4 production; such agents can be used, e.g., to redirect 20 the cytokine secretion profile of a Th2 cell to that of a Th1 cell.

A. Inhibitory Agents

According to a modulatory method of the invention, T-bet activity is inhibited in a cell by contacting the cell with an inhibitory agent. Inhibitory agents of the invention 25 can be, for example, intracellular binding molecules that act to inhibit the expression or activity of T-bet. As used herein, the term "intracellular binding molecule" is intended to include molecules that act intracellularly to inhibit the expression or activity of a protein by binding to the protein itself, to a nucleic acid (e.g., an mRNA molecule) that encodes the protein or to a target with which the protein normally interacts (e.g., to a 30 DNA target sequence to which T-bet binds). Examples of intracellular binding molecules, described in further detail below, include antisense T-bet nucleic acid

molecules (e.g., to inhibit translation of T-bet mRNA), intracellular anti-T-bet antibodies (e.g., to inhibit the activity of T-bet protein) and dominant negative mutants of the T-bet protein.

In one embodiment, an inhibitory agent of the invention is an antisense nucleic acid molecule that is complementary to a gene encoding T-bet or to a portion of said gene, or a recombinant expression vector encoding said antisense nucleic acid molecule. The use of antisense nucleic acids to downregulate the expression of a particular protein in a cell is well known in the art (see e.g., Weintraub, H. *et al.*, *Antisense RNA as a molecular tool for genetic analysis, Reviews - Trends in Genetics*, Vol. 1(1) 1986; 5 Askari, F.K. and McDonnell, W.M. (1996) *N. Eng. J. Med.* 334:316-318; Bennett, M.R. and Schwartz, S.M. (1995) *Circulation* 92:1981-1993; Mercola, D. and Cohen, J.S. (1995) *Cancer Gene Ther.* 2:47-59; Rossi, J.J. (1995) *Br. Med. Bull.* 51:217-225; Wagner, R.W. (1994) *Nature* 372:333-335). An antisense nucleic acid molecule 10 comprises a nucleotide sequence that is complementary to the coding strand of another nucleic acid molecule (e.g., an mRNA sequence) and accordingly is capable of hydrogen bonding to the coding strand of the other nucleic acid molecule. Antisense sequences complementary to a sequence of an mRNA can be complementary to a sequence found in the coding region of the mRNA, the 5' or 3' untranslated region of the mRNA or a region bridging the coding region and an untranslated region (e.g., at the junction of the 15 5' untranslated region and the coding region). Furthermore, an antisense nucleic acid can be complementary in sequence to a regulatory region of the gene encoding the mRNA, for instance a transcription initiation sequence or regulatory element. Preferably, an antisense nucleic acid is designed so as to be complementary to a region preceding or spanning the initiation codon on the coding strand or in the 3' untranslated 20 region of an mRNA. An antisense nucleic acid for inhibiting the expression of T-bet protein in a cell can be designed based upon the nucleotide sequence encoding the T-bet protein (e.g., SEQ ID NO: 1 or 3), constructed according to the rules of Watson and Crick base pairing.

25 An antisense nucleic acid can exist in a variety of different forms. For example, the antisense nucleic acid can be an oligonucleotide that is complementary to only a portion of a T-bet gene. An antisense oligonucleotides can be constructed using

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chemical synthesis procedures known in the art. An antisense oligonucleotide can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.* phosphorothioate derivatives and acridine substituted nucleotides can be used.

To inhibit T-bet expression in cells in culture, one or more antisense oligonucleotides can be added to cells in culture media, typically at about 200 µg oligonucleotide/ml.

Alternatively, an antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, nucleic acid transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest). Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the expression of the antisense RNA molecule in a cell of interest, for instance promoters and/or enhancers or other regulatory sequences can be chosen which direct constitutive, tissue specific or inducible expression of antisense RNA. For example, for inducible expression of antisense RNA, an inducible eukaryotic regulatory system, such as the Tet system (*e.g.*, as described in Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89**:5547-5551; Gossen, M. *et al.* (1995) *Science* **268**:1766-1769; PCT Publication No. WO 94/29442; and PCT Publication No. WO 96/01313) can be used. The antisense expression vector is prepared as described above for recombinant expression vectors, except that the cDNA (or portion thereof) is cloned into the vector in the antisense orientation. The antisense expression vector can be in the form of, for example, a recombinant plasmid, phagemid or attenuated virus. The antisense expression vector is introduced into cells using a standard transfection technique, as described above for recombinant expression vectors.

In another embodiment, an antisense nucleic acid for use as an inhibitory agent is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region (for reviews on ribozymes see *e.g.*, Ohkawa, J. *et al.* (1995) *J. Biochem.* **118**:251-258; Sigurdsson, S.T. and Eckstein, F. (1995) *Trends Biotechnol.* **13**:286-289; Rossi, J.J. (1995) *Trends Biotechnol.* **13**:301-306; Kiehn topf,

M. *et al.* (1995) *J. Mol. Med.* 73:65-71). A ribozyme having specificity for T-bet mRNA can be designed based upon the nucleotide sequence of the T-bet cDNA. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the base sequence of the active site is complementary to the base sequence to be cleaved in a 5 T-bet mRNA. See for example U.S. Patent Nos. 4,987,071 and 5,116,742, both by Cech *et al.* Alternatively, T-bet mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See for example Bartel, D. and Szostak, J.W. (1993) *Science* 261: 1411-1418.

Another type of inhibitory agent that can be used to inhibit the expression and/or 10 activity of T-bet in a cell is an intracellular antibody specific for the T-bet protein. The use of intracellular antibodies to inhibit protein function in a cell is known in the art (see e.g., Carlson, J. R. (1988) *Mol. Cell. Biol.* 8:2638-2646; Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Werge, T.M. *et al.* (1990) *FEBS Letters* 274:193-198; Carlson, J.R. (1993) *Proc. Natl. Acad. Sci. USA* 90:7427-7428; Marasco, W.A. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893; Biocca, S. *et al.* (1994) *Bio/Technology* 12:396-399; Chen, S-Y. *et al.* (1994) *Human Gene Therapy* 5:595-601; Duan, L *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5075-5079; Chen, S-Y. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5932-5936; Beerli, R.R. *et al.* (1994) *J. Biol. Chem.* 269:23931-23936; Beerli, R.R. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:666-672; Mhashilkar, A.M. *et al.* 15 (1995) *EMBO J.* 14:1542-1551; Richardson, J.H. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:3137-3141; PCT Publication No. WO 94/02610 by Marasco *et al.*; and PCT Publication No. WO 95/03832 by Duan *et al.*).

To inhibit protein activity using an intracellular antibody, a recombinant 20 expression vector is prepared which encodes the antibody chains in a form such that, upon introduction of the vector into a cell, the antibody chains are expressed as a functional antibody in an intracellular compartment of the cell. For inhibition of T-bet activity according to the inhibitory methods of the invention, an intracellular antibody that specifically binds the T-bet protein is expressed in the cytoplasm of the cell. To 25 prepare an intracellular antibody expression vector, antibody light and heavy chain cDNAs encoding antibody chains specific for the target protein of interest, e.g., T-bet, are isolated, typically from a hybridoma that secretes a monoclonal antibody specific for 30

the T-bet protein. Hybridomas secreting anti- T-bet monoclonal antibodies, or recombinant anti-T-bet monoclonal antibodies, can be prepared as described above.

Once a monoclonal antibody specific for T-bet protein has been identified (e.g., either a hybridoma-derived monoclonal antibody or a recombinant antibody from a

5 combinatorial library). DNAs encoding the light and heavy chains of the monoclonal antibody are isolated by standard molecular biology techniques. For hybridoma derived antibodies, light and heavy chain cDNAs can be obtained, for example, by PCR amplification or cDNA library screening. For recombinant antibodies, such as from a phage display library, cDNA encoding the light and heavy chains can be recovered from 10 the display package (e.g., phage) isolated during the library screening process.

Nucleotide sequences of antibody light and heavy chain genes from which PCR primers or cDNA library probes can be prepared are known in the art. For example, many such sequences are disclosed in Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services,

15 NIH Publication No. 91-3242 and in the "Vbase" human germline sequence database.

Once obtained, the antibody light and heavy chain sequences are cloned into a recombinant expression vector using standard methods. To allow for cytoplasmic expression of the light and heavy chains, the nucleotide sequences encoding the hydrophobic leaders of the light and heavy chains are removed. An intracellular

20 antibody expression vector can encode an intracellular antibody in one of several different forms. For example, in one embodiment, the vector encodes full-length antibody light and heavy chains such that a full-length antibody is expressed intracellularly. In another embodiment, the vector encodes a full-length light chain but only the VH/CH1 region of the heavy chain such that a Fab fragment is expressed

25 intracellularly. In the most preferred embodiment, the vector encodes a single chain antibody (scFv) wherein the variable regions of the light and heavy chains are linked by a flexible peptide linker (e.g., (Gly₄Ser)₃) and expressed as a single chain molecule. To inhibit T-bet activity in a cell, the expression vector encoding the anti-T-bet intracellular antibody is introduced into the cell by standard transfection methods, as discussed

30 hereinbefore.

Yet another form of an inhibitory agent of the invention is an inhibitory form of T-bet, also referred to herein as a dominant negative inhibitor, e.g., a form of T-bet in which the tyrosine phosphorylation sites have been mutated or, for example, a mutated form of T-bet in which the transactivation domain has been removed. Such dominant negative T-bet proteins can be expressed in cells using a recombinant expression vector encoding the T-bet protein, which is introduced into the cell by standard transfection methods. To express a mutant form of T-bet lacking tyrosine phosphorylation sites or a transactivation domain, nucleotide sequences encoding a transactivation domain of T-bet are mutated or removed from the *T-bet* coding sequences by standard recombinant DNA techniques. The truncated DNA is inserted into a recombinant expression vector, which is then introduced into a cell to allow for expression of the altered form of T-bet, in the cell.

Other inhibitory agents that can be used to inhibit the activity of a T-bet protein are chemical compounds that directly inhibit T-bet activity or inhibit the interaction between T-bet and target DNA or another protein. Such compounds can be identified using screening assays that select for such compounds, as described in detail above.

B. *Stimulatory Agents*

According to a modulatory method of the invention, T-bet activity is stimulated in a cell by contacting the cell with a stimulatory agent. Examples of such stimulatory agents include active T-bet protein and nucleic acid molecules encoding T-bet that are introduced into the cell to increase T-bet activity in the cell. A preferred stimulatory agent is a nucleic acid molecule encoding a T-bet protein, wherein the nucleic acid molecule is introduced into the cell in a form suitable for expression of the active T-bet protein in the cell. To express a T-bet protein in a cell, typically a T-bet-encoding DNA is first introduced into a recombinant expression vector using standard molecular biology techniques, as described herein. A T-bet-encoding DNA can be obtained, for example, by amplification using the polymerase chain reaction (PCR), using primers based on the T-bet nucleotide sequence. Following isolation or amplification of T-bet-encoding DNA, the DNA fragment is introduced into an expression vector and transfected into target cells by standard methods, as described herein.

Other stimulatory agents that can be used to stimulate the activity of a T-bet protein are chemical compounds that stimulate T-bet activity in cells, such as compounds that directly stimulate T-bet protein and compounds that promote the interaction between T-bet and target DNA or other proteins. Such compounds can be 5 identified using screening assays that select for such compounds, as described in detail above.

The modulatory methods of the invention can be performed *in vitro* (e.g., by culturing the cell with the agent or by introducing the agent into cells in culture) or, alternatively, *in vivo* (e.g., by administering the agent to a subject or by introducing the 10 agent into cells of a subject, such as by gene therapy). For practicing the modulatory method *in vitro*, cells can be obtained from a subject by standard methods and incubated (i.e., cultured) *in vitro* with a modulatory agent of the invention to modulate T-bet activity in the cells. For example, peripheral blood mononuclear cells (PBMCs) can be obtained from a subject and isolated by density gradient centrifugation, e.g., with 15 Ficoll/Hypaque. Specific cell populations can be depleted or enriched using standard methods. For example, T cells can be enriched for example, by positive selection using antibodies to T cell surface markers, for example by incubating cells with a specific primary monoclonal antibody (mAb), followed by isolation of cells that bind the mAb using magnetic beads coated with a secondary antibody that binds the primary mAb. 20 Specific cell populations can also be isolated by fluorescence activated cell sorting according to standard methods. If desired, cells treated *in vitro* with a modulatory agent of the invention can be readministered to the subject. For administration to a subject, it may be preferable to first remove residual agents in the culture from the cells before 25 administering them to the subject. This can be done for example by a Ficoll/Hypaque gradient centrifugation of the cells. For further discussion of *ex vivo* genetic modification of cells followed by readministration to a subject, see also U.S. Patent No. 5,399,346 by W.F. Anderson *et al.*

For practicing the modulatory method *in vivo* in a subject, the modulatory agent can be administered to the subject such that T-bet activity in cells of the subject is 30 modulated. The term "subject" is intended to include living organisms in which an

immune response can be elicited. Preferred subjects are mammals. Examples of subjects include humans, monkeys, dogs, cats, mice, rats, cows, horses, goats and sheep.

For stimulatory or inhibitory agents that comprise nucleic acids (including recombinant expression vectors encoding T-bet protein, antisense RNA, intracellular antibodies or dominant negative inhibitors), the agents can be introduced into cells of the subject using methods known in the art for introducing nucleic acid (e.g., DNA) into cells *in vivo*. Examples of such methods encompass both non-viral and viral methods, including:

Direct Injection: Naked DNA can be introduced into cells *in vivo* by directly 10 injecting the DNA into the cells (see e.g., Acsadi *et al.* (1991) *Nature* 332:815-818; Wolff *et al.* (1990) *Science* 247:1465-1468). For example, a delivery apparatus (e.g., a "gene gun") for injecting DNA into cells *in vivo* can be used. Such an apparatus is commercially available (e.g., from BioRad).

Cationic Lipids: Naked DNA can be introduced into cells *in vivo* by complexing 15 the DNA with cationic lipids or encapsulating the DNA in cationic liposomes. Examples of suitable cationic lipid formulations include N-[1-(2,3-dioleyloxy)propyl]N,N,N-triethylammonium chloride (DOTMA) and a 1:1 molar ratio of 1,2-dimyristyloxy-propyl-3-dimethylhydroxyethylammonium bromide (DMRIE) and dioleoyl phosphatidylethanolamine (DOPE) (see e.g., Logan, J.J. *et al.* (1995) *Gene Therapy* 2:38-49; San, H. *et al.* (1993) *Human Gene Therapy* 4:781-788).

Receptor-Mediated DNA Uptake: Naked DNA can also be introduced into cells *in vivo* by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C.H. (1988) *J. Biol. Chem.* 263:14621; Wilson *et al.* (1992) *J. Biol. Chem.* 267:963-967; and U.S. Patent No. 25 5,166,320). Binding of the DNA-ligand complex to the receptor facilitates uptake of the DNA by receptor-mediated endocytosis. A DNA-ligand complex linked to adenovirus capsids which naturally disrupt endosomes, thereby releasing material into the cytoplasm can be used to avoid degradation of the complex by intracellular lysosomes (see for example Curiel *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8850; Cristiano *et al.* 30 (1993) *Proc. Natl. Acad. Sci. USA* 90:2122-2126).

Retroviruses: Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). A recombinant retrovirus can be constructed having a nucleotide sequences of interest incorporated into the retroviral genome. Additionally, portions of the retroviral genome can be removed to render the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. *et al.* (eds.) Greene Publishing Associates, (1989). Sections 9.10-9.14 and other standard laboratory manuals.

Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include ψ Crip, ψ Cre, ψ 2 and ψ Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis, *et al.* (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury *et al.* (1991) *Science* 254:1802-1805; van Beusechem *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay *et al.* (1992) *Human Gene Therapy* 3:641-647; Dai *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu *et al.* (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Retroviral vectors require target cell division in order for the retroviral genome (and foreign nucleic acid inserted into it) to be integrated into the host genome to stably introduce nucleic acid into the cell. Thus, it may be necessary to stimulate replication of the target cell.

Adenoviruses: The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner *et al.* (1988) *BioTechniques* 6:616; Rosenfeld *et al.* (1991) *Science* 252:431-434; and Rosenfeld *et al.* (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld *et al.* (1992) cited 10 *supra*), endothelial cells (Lemarchand *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding 15 potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner *et al.* cited *supra*; Haj-Ahmad and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral 20 vectors currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80 % of the adenoviral genetic material.

Adeno-Associated Viruses: Adeno-associated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review 25 see Muzyczka *et al.* *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte *et al.* (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski *et al.* (1989) *J. Virol.* 63:3822-3828; and McLaughlin *et al.* (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 30 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin *et al.* (1985)

Mol. Cell. Biol. 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat *et al.* (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin *et al.* (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford *et al.* (1988) *Mol. Endocrinol.* 2:32-39; Tratschin *et al.* (1984) *J. Virol.* 51:611-619; and Flotte *et al.* (1993) *J. Biol. Chem.* 268:3781-3790).

The efficacy of a particular expression vector system and method of introducing nucleic acid into a cell can be assessed by standard approaches routinely used in the art. For example, DNA introduced into a cell can be detected by a filter hybridization 10 technique (e.g., Southern blotting) and RNA produced by transcription of introduced DNA can be detected, for example, by Northern blotting, RNase protection or reverse transcriptase-polymerase chain reaction (RT-PCR). The gene product can be detected by an appropriate assay, for example by immunological detection of a produced protein, such as with a specific antibody, or by a functional assay to detect a functional activity 15 of the gene product.

In a preferred embodiment, a retroviral expression vector encoding T-bet is used to express T-bet protein in cells *in vivo*, to thereby stimulate T-bet protein activity *in vivo*. Such retroviral vectors can be prepared according to standard methods known in the art (discussed further above).

20 A modulatory agent, such as a chemical compound, can be administered to a subject as a pharmaceutical composition. Such compositions typically comprise the modulatory agent and a pharmaceutically acceptable carrier. As used herein the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption 25 delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions. Pharmaceutical 30 compositions can be prepared as described above in subsection IV.

The identification of T-bet as a key regulator of the development of Th1 cells described herein, and in the repression of the Th2 phenotype, allows for selective manipulation of T cell subsets in a variety of clinical situations using the modulatory methods of the invention. The stimulatory methods of the invention (*i.e.*, methods that 5 use a stimulatory agent to enhance T-bet activity) result in production of IFN-gamma, with concomitant promotion of a Th1 response and downregulation of both IL-2 and IL-4, thus downmodulating the Th2 response. In contrast, the inhibitory methods of the invention (*i.e.*, methods that use an inhibitory agent to downmodulate T-bet activity) inhibit the production of IFN-gamma, with concomitant downregulation of a Th1 10 response and promotion of a Th2 response. Thus, to treat a disease condition wherein a Th1 response is beneficial, a stimulatory method of the invention is selected such that Th1 responses are promoted while downregulating Th2 responses. Alternatively, to treat a disease condition wherein a Th2 response is beneficial, an inhibitory method of the invention is selected such that Th1 responses are downregulated while promoting Th2 15 responses. Application of the methods of the invention to the treatment of disease conditions may result in cure of the condition, a decrease in the type or number of symptoms associated with the condition, either in the long term or short term (*i.e.*, amelioration of the condition) or simply a transient beneficial effect to the subject.

Numerous disease conditions associated with a predominant Th1 or Th2-type 20 response have been identified and could benefit from modulation of the type of response mounted in the individual suffering from the disease condition. Application of the immunomodulatory methods of the invention to such diseases is described in further detail below.

25 A. *Allergies*

Allergies are mediated through IgE antibodies whose production is regulated by the activity of Th2 cells and the cytokines produced thereby. In allergic reactions, IL-4 is produced by Th2 cells, which further stimulates production of IgE antibodies and activation of cells that mediate allergic reactions, *i.e.*, mast cells and basophils. IL-4 30 also plays an important role in eosinophil mediated inflammatory reactions. Accordingly, the stimulatory methods of the invention can be used to inhibit the

production of Th2-associated cytokines, and in particular IL-4, in allergic patients as a means to downregulate production of pathogenic IgE antibodies. A stimulatory agent may be directly administered to the subject or cells (*e.g.*, Thp cells or Th2 cells) may be obtained from the subject, contacted with a stimulatory agent *ex vivo*, and

5 readministered to the subject. Moreover, in certain situations it may be beneficial to coadminister to the subject the allergen together with the stimulatory agent or cells treated with the stimulatory agent to inhibit (*e.g.*, desensitize) the allergen-specific response. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines

10 (*e.g.*, anti-IL-4 antibodies), to the allergic subject in amounts sufficient to further stimulate a Th1-type response.

B. *Cancer*

The expression of Th2-promoting cytokines has been reported to be elevated in

15 cancer patients (see *e.g.*, Yamamura, M., *et al.* (1993) *J. Clin. Invest.* 91:1005-1010; Pisa, P., *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7708-7712) and malignant disease is often associated with a shift from Th1 type responses to Th2 type responses along with a worsening of the course of the disease. Accordingly, the stimulatory methods of the invention can be used to inhibit the production of Th2-associated cytokines in cancer

20 patients, as a means to counteract the Th1 to Th2 shift and thereby promote an ongoing Th1 response in the patients to ameliorate the course of the disease. The stimulatory method can involve either direct administration of a stimulatory agent to a subject with cancer or *ex vivo* treatment of cells obtained from the subject (*e.g.*, Thp or Th2 cells) with a stimulatory agent followed by readministration of the cells to the subject. The

25 treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (*e.g.*, anti-IL-4 antibodies), to the recipient in amounts sufficient to further stimulate a Th1-type response.

C. Infectious Diseases

The expression of Th2-promoting cytokines also has been reported to increase during a variety of infectious diseases, including HIV infection, tuberculosis, leishmaniasis, schistosomiasis, filarial nematode infection and intestinal nematode infection (see e.g.; Shearer, G.M. and Clerici, M. (1992) *Prog. Chem. Immunol.* 54:21-43; Clerici, M and Shearer, G.M. (1993) *Immunology Today* 14:107-111; Fauci, A.S. (1988) *Science* 239:617-623; Locksley, R. M. and Scott, P. (1992) *Immunoparasitology Today* 1:A58-A61; Pearce, E.J., et al. (1991) *J. Exp. Med.* 173:159-166; Grzych, J-M., et al. (1991) *J. Immunol.* 141:1322-1327; Kullberg, M.C., et al. (1992) *J. Immunol.* 148:3264-3270; Bancroft, A.J., et al. (1993) *J. Immunol.* 150:1395-1402; Pearlman, E., et al. (1993) *Infect. Immun.* 61:1105-1112; Else, K.J., et al. (1994) *J. Exp. Med.* 179:347-351) and such infectious diseases are also associated with a Th1 to Th2 shift in the immune response. Accordingly, the stimulatory methods of the invention can be used to inhibit the production of Th2-associated cytokines in subjects with infectious diseases, as a means to counteract the Th1 to Th2 shift and thereby promote an ongoing Th1 response in the patients to ameliorate the course of the infection. The stimulatory method can involve either direct administration of an inhibitory agent to a subject with an infectious disease or *ex vivo* treatment of cells obtained from the subject (e.g., Thp or Th2 cells) with a stimulatory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (e.g., anti-IL-4 antibodies), to the recipient in amounts sufficient to further stimulate a Th1-type response.

25 D. Autoimmune Diseases

The inhibitory methods of the invention can be used therapeutically in the treatment of autoimmune diseases that are associated with a Th2-type dysfunction. Many autoimmune disorders are the result of inappropriate activation of T cells that are reactive against self tissue and that promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Modulation of T helper-type responses can have an effect on the course of the autoimmune disease. For example, in

experimental allergic encephalomyelitis (EAE), stimulation of a Th2-type response by administration of IL-4 at the time of the induction of the disease diminishes the intensity of the autoimmune disease (Paul, W.E., *et al.* (1994) *Cell* 76:241-251). Furthermore, recovery of the animals from the disease has been shown to be associated with an 5 increase in a Th2-type response as evidenced by an increase of Th2-specific cytokines (Koury, S. J., *et al.* (1992) *J. Exp. Med.* 176:1355-1364). Moreover, T cells that can suppress EAE secrete Th2-specific cytokines (Chen, C., *et al.* (1994) *Immunity* 1:147-154). Since stimulation of a Th2-type response in EAE has a protective effect against the disease, stimulation of a Th2 response in subjects with multiple sclerosis (for which 10 EAE is a model) is likely to be beneficial therapeutically. The inhibitory methods of the invention can be used to effect such a decrease.

Similarly, stimulation of a Th2-type response in type I diabetes in mice provides a protective effect against the disease. Indeed, treatment of NOD mice with IL-4 (which promotes a Th2 response) prevents or delays onset of type I diabetes that normally 15 develops in these mice (Rapoport, M.J., *et al.* (1993) *J. Exp. Med.* 178:87-99). Thus, stimulation of a Th2 response in a subject suffering from or susceptible to diabetes may ameliorate the effects of the disease or inhibit the onset of the disease.

Yet another autoimmune disease in which stimulation of a Th2-type response may be beneficial is rheumatoid arthritis (RA). Studies have shown that patients with 20 rheumatoid arthritis have predominantly Th1 cells in synovial tissue (Simon, A.K., *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:8562-8566). By stimulating a Th2 response in a subject with RA, the detrimental Th1 response can be concomitantly downmodulated to thereby ameliorate the effects of the disease.

Accordingly, the inhibitory methods of the invention can be used to stimulate 25 production of Th2-associated cytokines in subjects suffering from, or susceptible to, an autoimmune disease in which a Th2-type response is beneficial to the course of the disease. The inhibitory method can involve either direct administration of an inhibitory agent to the subject or *ex vivo* treatment of cells obtained from the subject (*e.g.*, Thp, Th1 cells, B cells, non-lymphoid cells) with an inhibitory agent followed by 30 readministration of the cells to the subject. The treatment may be further enhanced by administering other Th2-promoting agents, such as IL-4 itself or antibodies to Th1-

associated cytokines to the subject in amounts sufficient to further stimulate a Th2-type response.

In contrast to the autoimmune diseases described above in which a Th2 response is desirable, other autoimmune diseases may be ameliorated by a Th1-type response.

5 Such diseases can be treated using a stimulatory agent of the invention (as described above for cancer and infectious diseases). The treatment may be further enhanced by administrating a Th1-promoting cytokine (e.g., IFN- γ) to the subject in amounts sufficient to further stimulate a Th1-type response.

The efficacy of agents for treating autoimmune diseases can be tested in the 10 above described animal models of human diseases (e.g., EAE as a model of multiple sclerosis and the NOD mice as a model for diabetes) or other well characterized animal models of human autoimmune diseases. Such animal models include the *mlr/lpr/lpr* mouse as a model for lupus erythematosus, murine collagen-induced arthritis as a model for rheumatoid arthritis, and murine experimental myasthenia gravis (see Paul ed., 15 *Fundamental Immunology*, Raven Press, New York, 1989, pp. 840-856). A modulatory (i.e., stimulatory or inhibitory) agent of the invention is administered to test animals and the course of the disease in the test animals is then monitored by the standard methods for the particular model being used. Effectiveness of the modulatory agent is evidenced by amelioration of the disease condition in animals treated with the agent as compared to 20 untreated animals (or animals treated with a control agent).

Non-limiting examples of autoimmune diseases and disorders having an 25 autoimmune component that may be treated according to the invention include diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, myasthenia gravis, systemic lupus erythematosus, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren's Syndrome, including keratoconjunctivitis sicca secondary to Sjögren's Syndrome, alopecia areata, allergic responses due to 30 arthropod bite reactions, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions,

leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis,

5 Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Crohn's disease, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis.

E. *Transplantation*

10 While graft rejection or graft acceptance may not be attributable exclusively to the action of a particular T cell subset (*i.e.*, Th1 or Th2 cells) in the graft recipient (for a discussion see Dallman, M.J. (1995) *Curr. Opin. Immunol.* 7:632-638), numerous studies have implicated a predominant Th2 response in prolonged graft survival or a predominant Th2 response in graft rejection. For example, graft acceptance has been

15 associated with production of a Th2 cytokine pattern and/or graft rejection has been associated with production of a Th1 cytokine pattern (see *e.g.*, Takeuchi, T. *et al.* (1992) *Transplantation* 53:1281-1291; Tzakis, A.G. *et al.* (1994) *J. Pediatr. Surg.* 29:754-756; Thai, N.L. *et al.* (1995) *Transplantation* 59:274-281). Additionally, adoptive transfer of cells having a Th2 cytokine phenotype prolongs skin graft survival (Maeda, H. *et al.* (1994) *Int. Immunol.* 6:855-862) and reduces graft-versus-host disease (Fowler, D.H. *et al.* (1994) *Blood* 84:3540-3549; Fowler, D.H. *et al.* (1994) *Prog. Clin. Biol. Res.* 389:533-540). Still further, administration of IL-4, which promotes Th2 differentiation, prolongs cardiac allograft survival (Levy, A.E. and Alexander, J.W. (1995) *Transplantation* 60:405-406), whereas administration of IL-12 in combination with anti-

20 IL-10 antibodies, which promotes Th1 differentiation, enhances skin allograft rejection (Gorczyński, R.M. *et al.* (1995) *Transplantation* 60:1337-1341).

Accordingly, the inhibitory methods of the invention can be used to stimulate production of Th2-associated cytokines in transplant recipients to prolong survival of the graft. The inhibitory methods can be used both in solid organ transplantation and in

25 bone marrow transplantation (*e.g.*, to inhibit graft-versus-host disease). The inhibitory method can involve either direct administration of an inhibitory agent to the transplant

recipient or *ex vivo* treatment of cells obtained from the subject (*e.g.*, Thp, Th1 cells, B cells, non-lymphoid cells) with an inhibitory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th2-promoting agents, such as IL-4 itself or antibodies to Th1-associated cytokines, to the 5 recipient in amounts sufficient to further inhibit a Th2-type response.

In addition to the foregoing disease situations, the modulatory methods of the invention also are useful for other purposes. For example, the stimulatory methods of the invention (*i.e.*, methods using a stimulatory agent) can be used to stimulate production of Th1-promoting cytokines (*e.g.*, interferon-gamma) *in vitro* for commercial 10 production of these cytokines (*e.g.*, cells can be contacted with the stimulatory agent *in vitro* to stimulate interferon-gamma production and the interferon-gamma can be recovered from the culture supernatant, further purified if necessary, and packaged for commercial use).

Furthermore, the modulatory methods of the invention can be applied to 15 vaccinations to promote either a Th1 or a Th2 response to an antigen of interest in a subject. That is, the agents of the invention can serve as adjuvants to direct an immune response to a vaccine either to a Th1 response or a Th2 response. For example, to promote an antibody response to an antigen of interest (*i.e.*, for vaccination purposes), the antigen and an inhibitory agent of the invention can be coadministered to a subject to 20 promote a Th2 response to the antigen in the subject, since Th2 responses provide efficient B cell help and promote IgG1 production. Alternatively, to promote a cellular immune response to an antigen of interest, the antigen and a stimulatory agent of the invention can be coadministered to a subject to promote a Th1 response to the antigen in a subject, since Th1 responses favor the development of cell-mediated immune 25 responses (*e.g.*, delayed hypersensitivity responses). The antigen of interest and the modulatory agent can be formulated together into a single pharmaceutical composition or in separate compositions. In a preferred embodiment, the antigen of interest and the modulatory agent are administered simultaneously to the subject. Alternatively, in certain situations it may be desirable to administer the antigen first and then the 30 modulatory agent or vice versa (for example, in the case of an antigen that naturally evokes a Th1 response, it may be beneficial to first administer the antigen alone to

stimulate a Th1 response and then administer an inhibitory agent, alone or together with a boost of antigen, to shift the immune response to a Th2 response).

This invention is further illustrated by the following example, which should not be construed as limiting. The contents of all references, patents and published patent 5 applications cited throughout this application are hereby incorporated by reference. Additionally, all nucleotide and amino acid sequences deposited in public databases referred to herein are also hereby incorporated by reference.

A nucleic acid molecule comprising a mouse T-bet cDNA cloned into the EcoRI site of the pJG4-5 vector was deposited with the American Type Culture Collection 10 (Manassas, VA) on November 9, 1999 and assigned Deposit Number PTA-930. A nucleic acid molecule comprising a human T-bet cDNA (prepared from RNA from the human Th1 clone ROT-10) cloned into the PCR 2.1-TOPO vector was deposited with the American Type Culture Collection (Manassas, VA) on January 28, 2000 and assigned Deposit Number PTA-1339. Both deposits were made under the provisions of 15 the Budapest Treaty.

EXAMPLES

The following experimental procedures were used in the examples:

20 **Mice, Cell Lines, Cytokines, Antibodies and Plasmids**

BALB/c mice were obtained from Jackson Laboratories, DO11.10 TcR-transgenic mice (Jacobson, N.G., et al. 1995. *J. Exp. Med.* 181, 1755-1762), and MBP TcR-transgenic mice (Lafaille, J.J., 1994. *Cell* 78, 399-408.) have been described. Mice were used at 5-6 weeks of age. Cell lines and primary cells were maintained in complete 25 medium containing RPMI 1640 supplemented with 10% fetal calf serum (HyClone Laboratories), glutamine (2mM), penicillin (50 units/ml), streptomycin (50 μ g/ml), Hepes (100mM) and β -ME (50 μ M). Jurkat is a human Th1 lymphoma, EL4 a mouse Th0 thymoma, NK3.3 a human NK cell line (Ye, J., 1995. *J. Leuko. Biol.* 58, 225-233.; Kornbluth, J., 1982. *J. Immunol.* 129, 2831-2837), YT a human NK cell line 30 (Yodoi, J., 1985. *J. of Immuno.* 134, 1623-1630), AE7 a mouse Th1 clone, D10 a mouse

Th2 clone and M12 is a B cell lymphoma line. Recombinant IL-4 was obtained from DNAX, human rIL-2 was obtained from Chiron Corp., rIL-12 was obtained from Hoffman LaRoche, and rIL-18 was purchased from Peprotech, Inc. Monoclonal anti-IL-12, monoclonal anti-IFN- γ and monoclonal anti-IL-4 (11B11) were also used (Ohara, J. 5 and Paul, W.E. 1985. *Nature* 315, 333-336). Both the T-bet polyclonal antisera, produced in rabbits, and the mAb were raised against full-length recombinant bacterially produced T-bet. The mAb was produced by fusion of spleen cells from mice to the SP2/O-Ag14 myeloma and is of the IgG1 subtype. Expression plasmids included c-Maf (pMex-maf) (Ho, I-C., et al. 1996. *Cell* 85, 973-983.), NFATp (Hodge, M.R., et al. 10 1996. *Immunity* 4, 1-20) and p65, the latter two cloned into the pCDNA vector.

CD4+ T Cell Purification and In Vitro Cultures

CD4+ T cells were purified from lymph nodes (LN) by flow cytometry using PE-conjugated anti CD4 (RM4-4) (Pharmingen) and sorted using FACS (Mo Flo, 15 Becton Dickenson) to 98-99% purity. For in vitro activation 2×10^6 /ml CD4+ cells were resuspended in complete medium and activated with plate-bound 1 μ g/ml anti CD3 (2C11) and 2 μ g/ml anti CD28 (Pharmingen) for 3 days in the presence of 100 units/ml IL2. Cells were then split 1:4 in complete medium and cultured for 4 days in the presence of 100 units/ml IL2. On day 7 after primary stimulation, cells were harvested, 20 washed twice and restimulated at 1×10^6 cells/ml with 1 μ g/ml plate-bound anti CD3 for 1, 3 and 6 hours. For Th1 and Th2 differentiation cultures, non-transgenic or DO11.10 LN and spleen cells were pooled, resuspended in 1×10^6 cells/ml complete medium and cultured under Th1 (10mg/ml anti IL4 [11B11], 10ng/ml rIL12) or Th2 (10mg/ml anti IFN γ , 10ng/ml IL4) conditions with 1 μ g/ml plate-bound anti CD3. Cells were split 1:4 25 on day 3 with complete medium + 100u/ml IL2. On day 7, cells were restimulated with 1 μ g/ml anti CD3 for 4 hours and harvested for RNA preparation (Jacobson, N.G., et al. 1995. *J. Exp. Med.* 181, 1755-1762). Supernatants were taken at 24 hours to test for cytokines.

Northern and Western Blot Analysis

Total RNA was isolated from resting and stimulated cells using TRIZOL reagent (Gibco/BRL) and 10 μ g of each sample separated on 1.2% agarose 6% formaldehyde gels, transferred onto Genescreen membrane (NEN) in 20X SSC overnight and

5 covalently bound using a UV Stratalinker (Stratagene). Hybridization of blots was carried out at 42°C as described (Hodge, M.R., et al. 1996. *Immunity* 4, 1-20) using the following cDNA probes labeled with 32P: T-bet, γ -actin. Nuclear and cytoplasmic extracts for western blot analysis were prepared from AE7, D10 and NK3.3 cells. Nuclei were isolated as described (Dolmetsch, R.E., et al. 1997. *Nature* 386, 855-858).

10 Extracted proteins were separated by 8% PAGE followed by electrotransfer to nitrocellulose membranes and probed with a mAb specific for T-bet followed by horseradish peroxidase-conjugated goat anti-mouse IgG and enhanced chemiluminescence according to the instructions of the manufacturer (Amersham).

15 **Transient Transfection Assays**

EL4 and Jurkat cells were transfected using a Bio Rad electroporator (280V, 975 μ F) using 5 X 10 6 cells in 0.4 ml RPMI per transfection with 5 μ g reporter plasmid and 5-10 μ g expression plasmid. Luciferase assays were performed after 24 hrs with the luciferase activity in 20% of each sample measured as per instructions (Promega). The

20 IFN- γ reporter-luciferase construct is derived from the plasmid pB9 which contains the entire human IFN-gamma gene (P. Gray and D.V. Goeddel. 1982. *Nature*. 298:859). The pGL2 luciferase gene was inserted into the first exon of pB9. IL-2-promoter-reporter construct The IL-4 promoter reporter construct, IL-4Luc, contains 807 bp upstream of the murine IL-4 gene.

25

Retroviral Constructs and Transduction

The GFP-RV bicistronic vector has been described (Ouyang, W., et al. 1998. *Immunity* 9:745-755) as has the Phoenix-Eco packaging cell line (Kinoshita, S., et al. 1998. *Cell* 95, 595-604). The GFP-RV vector was constructed by inserting the

30 encephalomyocarditis virus internal ribosomal entry sequence (IRES) and the GFP allele

into the MSCV2.2 retroviral vector (Ouyang, W., et al. 1998. *Immunity* 9:745-755) or IL-2-MSCV vector. Both vectors express two cDNAs. T-bet and the cDNA encoding GFP, simultaneously using an IRES to initiate translation of each mRNA separately. Transfection of the packaging cell line and retroviral transductions of primary T cells 5 were performed essentially as described (Ouyang, W., et al. 1998. *Immunity* 9:745-755).

Intracellular cytokine staining and FACS Analysis

Intracellular staining for cytokines was performed as described (Ouyang, W., et al. 1998. *Immunity* 9:745-755). Primary transgenic or non-transgenic T cells that had 10 been infected with retrovirus for various time periods as indicated were restimulated with PMA (50 ng/ml and ionomycin (1 uM) for 2 hours and 10 ug/ml Brefeldin A added for an additional 2 hours.

Example 1. Cloning of a novel transcription factor, T-bet

15 Since the Th1-specific region of the IL-2 promoter had been well localized (Brombacher, F., et al. 1994. *Int. Immunol.* 6:189-197.; Rooney, J., et al. 1995. *Mol. Cell. Biol.* 15, 6299-6310; Lederer, J.A., et al. 1994. *J. Immunol.* 152, 77-86; Durand, D., et al. 1988. *Mol. Cell. Biol.* 8, 1715-1724; Hoyos, B., et al. 1989. *Science* 244, 457-450), a yeast one hybrid approach using an IL-2 promoter-reporter and a cDNA library 20 made from the OF6 Th1 clone was chosen to identify Th1 specific transcription factors. To validate this approach, the Th2-specific region of the IL-4 promoter was expressed in yeast and demonstrated to be transactivated by the introduction of c-Maf, but not by several other transcription factors (eg NFAT). C-Maf transactivation did not occur when the c-Maf response element (MARE) was mutated. Thus, the yeast one hybrid approach 25 was utilized.

The EGY48 yeast strain was stably integrated with the IL-2 promoter/histidine construct and transformed with a cDNA library made from an anti-CD3 activated Th1 cell clone, OF6. Of 5.6×10^6 clones screened, 488 were positive in primary screening. Of the 210 clones tested during the secondary screen, 72 proved to be specific for the IL- 30 2 promoter. To reduce the number of positive clones, we hybridized the yeast clone cDNA with cDNAs that were differentially expressed in Th1 and Th2 cell lines. These

Th1-Th2 and Th2-Th1 cDNAs were made using the Clontech PCR select kit, radiolabeled and initially used in a pilot experiment to screen the 16 most strongly positive yeast clones. Of those 16 clones, 8 were positive with the Th1 (PL17) specific cDNA product probe and not with the Th2 (D10) specific cDNA product probe.

5 Representational difference analysis (RDA; e.g., Lisitsyn. 1993. *Science*. 259:946; O'Neill and Sinclair. 1997. *Nucleic Acids Res.* 25:2681; Hubank and Schatz. 1994. *Nucleic Acids Research*. 22:5640; Welford et al. 1998. *Nucleic Acids Research*. 26:3059) with Th1-Th2 probe on 16 positive clones with control hybridization of the probe to IL-2, IFN-gamma and IL-4 was performed. The specificity of the Th1 and Th2

10 subtracted cDNA probes is demonstrated by their detection of IL-2 and IFN- γ versus IL-4 respectively.

Restriction enzyme analyses and sequencing data revealed that all 8 of the clones were related. They fell into three groupings based on differences in the 5' and 3' untranslated regions. each of these categories representing an independent cDNA

15 15 molecule. Comparing the sequence of these clones with the NCBI GenBank Sequence Database yielded homology with the T-box family of transcription factors. Figure 1 shown the nucleotide and amino acid sequences of T-bet.

Example 2. T-bet shares a region of homology with the T-box family members T-brain and eomesodermin

Brachyury or *T* is the founding member of a family of transcription factors that share a 200 amino acid DNA-binding domain called the T-box (reviewed in (Smith, J. 1997. *Current Opinion in Genetics & Development* 7, 474-480; Papaioannou, and Silver. 1998. *Bioessay*. 20:9; Meisler, M.H. 1997. *Mammalian Genome* 8, 799-800.).

25 The *Brachyury* (Greek for 'short tail') mutation was first described in 1927 in heterozygous mutant animals who had a short, slightly kinked tail (Herrmann, B.G., 1990. *Nature* 343, 617-622). There are now eight T-box genes in the mouse not including *Brachyury*. These include Tbx1-6, T-brain-1 (Tbr-1) and now, T-bet, each with a distinct and usually complex expression pattern. The T-box family of

30 transcription factors is defined by homology of family members in the DNA binding domain. The T-bet DNA binding domain (residues 138-327 of murine T-bet) is most

similar to the T-box domains of murine T-brain and *Xenopus* eomesodermin and thus places T-bet in the Tbr1 subfamily of the T-box gene family. The human homologue of the murine T-bet protein is approximately 88 % identical to the mouse T-bet. Figure 1A was derived using a Lipman-Pearson protein alignment (with G penalty set at 4 and gap 5 length penalty set at 12. The similarity index was calculated to be 86.6; the gap number2, the gap length5, and the consensus length 535). T-bet shares a region of homology with the T-box family members T-brain and eomesodermin. The murine T-bet DNA binding domain is most similar to the T-box domains of murine T-brain and *Xenopus* eomesodermin. There is approximately 69% amino acid identity between the 10 three T-box regions. T-bet bears no sequence homology to other T-box family members outside of the T-box domain.

Example 3. T-bet binds to and transactivates consensus T-box sites and has functionally important domains that map to both 5' and 3' regions

15 Recombinant T-bet protein binds to consensus T-box sites and to the T-bet site in the IL-2 promoter, and a complex present in nuclear extracts from anti-CD3-stimulated AE7 Th1 cells binds specifically to a consensus (GGGAATTTCACACCTAGGTGAAATTCC) T-box oligonucleotide probe. To test for activity of T-bet in T cells, the following experiments were performed. Jurkat Th1 20 cells were cotransfected with T-bet and a luciferase reporter construct. Figure 2A shows the basal level (open bars) and the PMA (50ng/ml) plus ionomycin (1uM) induced (closed bars) promoter activity in Jurkat cells of a luciferase reporter construct containing a minimal thymidine kinase (TK) promoter with or without 4 copies of the consensus T-box site. Each reporter construct was co-transfected with empty pCDNA 25 vector or pCDNA containing the full-length T-bet cDNA as indicated in the figure. The data shown are representative of three independent experiments. Figure 2B shows Jurkat cells transiently transfected with the luciferase reporter construct containing the minimal TK promoter and multimerized consensus T-box sites and pCDNA vector containing the indicated regions of the T-bet cDNA diagrammed at the left of the bar graph. Luciferase 30 activity was measured 24 hours post-transfection. The experiment was repeated three times with similar results. The basal level (open bars) and the PMA (50ng/ml) plus

ionomycin (1uM) induced (closed bars) promoter activity obtained demonstrate that T-bet is active in T cells. and that its activity can be further increased upon stimulation.

Example 4. T-bet expression in T cells is restricted to the Th1 subset and regulated by signals transmitted via the TcR

T-bet was isolated from a Th1 cDNA library and a multiple organ Northern blot analysis revealed T-bet transcripts only in lung, thymus and in peripheral lymphoid organs.

Figure 3A shows that T-bet is preferentially expressed in double negative (DN) thymocytes, not in double positive (DP) or single positive (SP) cells. Northern blot analysis of total cellular RNA isolated from Th1 cell clones (AE7 and D1.1) or Th2 clones (D10 and CDC35) that were treated with media or with plate-bound anti-CD3 (2C11) for 6 hours revealed T-bet transcripts only in the Th1 clones. Total cellular RNA was isolated from Th1 cell clones (AE7 and D1.1) or Th2 clones (D10 and CDC35) that were treated with media or with plate-bound anti-CD3 (2C11) for 6 hours. Total RNA was also isolated from M12 (B-cell lymphoma and EL4 (T-cell thymoma) treated with media or with PMA (50ng/ml) and ionomycin (1uM) for 6 hours. Northern blot analysis was performed with 10 ug of total RNA per lane using standard procedures and probed using the full-length T-bet cDNA. T-bet is prefentially expressed in Th1 clones.

Further, the level of T-bet expression was augmented by signals transmitted via the TcR as evidenced by the induction of T-bet transcripts by anti-CD3. T-bet transcripts were not detected in M12, a B-cell lymphoma, in the Th1 lymphoma Jurkat or in EL4, a Th0-cell thymoma either when these cells were treated with media or with PMA (50ng/ml) and ionomycin (1uM) for 6 hours.

To determine protein levels of T-bet in primary T cells, DO11.10 TcR transgenic splenocytes were cultured under Th1 or Th2 polarizing conditions. At 72 hours the cells were expanded 3-fold in fresh medium with 200U/ml IL-2. On day 7 after primary stimulation, nuclear and cytosolic extracts were prepared from resting or PMA/ionomycin activated (1 hr) bulk culture DO11.10 Th1 and Th2 cells. Nuclear extracts were also prepared from resting M12, EL4, Jurkat, NK3.3, and YT cells. As shown in Figure 3C, among the cell lines, T-bet protein was present in YT cells only.

Figure 3C shows T-bet protein is restricted to Th1 cells and NK cells. Western blot analysis was performed on nuclear and cytosolic extracts prepared from resting or PMA/ionomycin activated (1 hr) bulk culture DO11.10 Th1 and Th2 cells as above. Briefly, DO11.10 Tcrtransgenic splenocytes were activated with OVA peptide (323-339) 5 at 3 x 106 cells/ml in the presence of 10 ng/ml IL-12 and 10 ug/ml anti-IL-4 (11B11) to promote Th1 phenotype development, or 10ng/ml IL-4 and 10 ug/ml anti-IFN-gamma to promote Th2 phenotype development. At 72 hours the cells were expanded 3-fold in fresh medium with 200 U/ml IL-2. On day 7 after primary stimulation, nuclear and cytosolic extracts were prepared from resting or PMA/ionomycin activate (1hr) bulk 10 culture DO11.10 Th1 and Th2 cells. Nuclear extracts were also prepared from resting M12 cells, EL4, Jurkat, NK3.3, and YT. 30 ug of nuclear and cytosolic extracts were separated by SDS-PAGE (8% gel), transferred to nitrocellulose, and probed with an anti T-bet antisera. In primary T cells, T-bet protein is selectively expressed in T cells driven along a Th1 but not a Th2 pathway, consistent with the Northern blot analysis of T cell 15 clones and primary T cells shown above.

A monoclonal antibody (mAb) specific for T-bet allowed the direct visualization of T-bet protein by FACS analysis. Figure 3D shows that T-bet can be visualised by FACS in activated AE7 Th1 cells. D10 (Th2) or AE7 (Th1) cells were treated with media or PMA (50ng/ml) plus ionomycin (1uM) for 2 hours and 2uM monensin for an 20 additional 3 hours. Cells were washed with PBS, fixed in 4% paraformaldehyde, permeabilized with 0.5% saponin, and stained with media (dashed line) or an IgG1 isotype control antibody (dotted line) or an affinity-purified anti-T-bet monoclonal antibody α 3D10 (solid line) followed by goat anti-mouse IgG1-PE staining. Cells were analyzed by flow cytometry on a FACSCalibur. Mouse monoclonal antibodies were 25 raised against bacterially produced T-bet. T-bet protein was not detectable in D10 cells, was present at low levels in unstimulated AE7 cells and was present at increased levels in stimulated AE7. Taken together, the experiments detailed here demonstrate that in T cells, T-bet is selectively expressed in Th1 cells where its level of expression is regulated by signals stemming from the TcR.

Example 5. T-bet expression correlates with IFN- γ induction in NK and B cells

The Th1-limited expression of T-bet coupled with its isolation by virtue of binding to a T-box site in the IL-2 promoter suggested that T-bet might activate the transcription of the IL-2 gene. However, it was puzzling that two IL-2-producing cell lines, Jurkat and EL4, did not express T-bet, while the NK cell line YT, which produces IFN- γ but not IL-2, did express T-bet. Further, preliminary experiments did not demonstrate transactivation of the IL-2 gene by T-bet, despite the presence of an excellent T-box site in the IL-2 promoter. Other Th1-specific cytokines include IFN- γ , TNF α and LT. The expression of T-bet correlated well with the expression of IFN- γ .
10 Further, a T-box site was found to be present in the third intron of the human IFN- γ gene. This was especially noteworthy since a Th1-specific DNaseI hypersensitivity site had recently been mapped to this region.

To examine the possibility that T-bet controlled the expression of the IFN- γ gene, the expression of T-bet and the expression of IFN- γ in cells other than Th1 cells was measured. IFN- γ is expressed in natural killer (NK) cells at low levels and is induced to high levels upon treatment with IL-2 and IL-12 (Kornbluth, J., et al. 1982. J. Immunol. 129:2831; Ye et al. 1995. J. Leuko. Biol. 58:225). Therefore, the NK3.3 cell line was treated for 24 h with IL-2, IL-12 and IL-2 plus IL-12, lysates prepared and western blot analysis performed with T-bet mAb as above. Figure 4b demonstrates coordinate induction of T-bet protein and secretion of IFN- γ in NK3.3 cells. The NK3.3 cell line was treated for 24 h with reagents, IL-2, IL-12 and IL-2 plus IL-12, known to induce IFN- γ in NK cells, lysates prepared and western blot analysis performed with T-bet mAb as above. ELISA was performed on supernatants harvested from the cells.
20

B cells, which do not produce IFN- γ at baseline, can be driven to produce large amounts of IFN- γ upon treatment with anti-CD40 antibody and a combination of IL-12 and IL-18 (Yoshimoto, T., 1997. Proc. Natl. Acad. Sci. USA 94, 3948-3953). Purified B cells were treated for 72h with anti-CD40 mAb, rIL-12 and rIL-18, RNA isolated and Northern blot performed using the T-bet cDNA as above. Figure 4A shows induction of T-bet mRNA in B cells treated with this combination of reagents, and the induction of IFN- γ transcripts in these cells was confirmed. In conclusion, while neither cell type
30

expresses T-bet constitutively, both NK3.3 cells and B cells can be induced to do so under conditions which also result in IFN- γ production. Thus, the pattern of expression of T-bet correlates well with the transcription of the IFN- γ gene.

5 **Example 6. T-bet transactivates the IFN- γ gene in Th cells**

Very little is yet known about the regulatory regions of the IFN- γ gene. In particular, the regions of the gene that direct its tissue-specific expression have not been identified *in vitro* or *in vivo*. It has been demonstrated that reporter constructs containing 500 bp or 3 kb of upstream sequence are expressed in both Th1 and Th2 cells

10 (Young, H.A., 1994. *J. of Immuno.* *153*, 3603-3610). ATF-2, NF κ B, AP-1 and Stat4 sites in the IFN- γ promoter or introns are thought to be functionally important, but clearly are not responsible for tissue-specific expression (Young, H.A., 1994. *J. of Immuno.* *153*, 3603-3610; Sica, A., 1997. *J. Biol. Chem.* *272*, 30412-30420; Penix, L., 1993. *J. Exp. Med.* *178*, 1483-1496; Penix, L.A., 1996. *J. Biol. Chem.* *271*, 31964-15 31972). Similarly, although Th1-preferential DNaseI hypersensitive sites have been noted both in the first and third introns, the relevant cis elements located in these introns have not been identified (Young, H.A., et al. 1994. *J. of Immuno.* *153*, 3603-3610; Agarwal, S. and Rao, A. 1998. *Immunity* *9*, 765-775). Therefore, a reporter construct containing the entire IFN- γ gene was utilized for these studies. The IFN- γ reporter gene

20 used includes 3 kb of upstream sequence, the entire coding sequence with all three introns, and 1.5 kb of downstream (Xu, X., et al. 1996. *Science* *273*, 794-796).

The activity of a luciferase reporter construct containing 9 kb of the IFN-gamma gene in the Jurkat human Th1 lymphoma and the mouse EL4 Th0 thymoma was tested. Each reporter construct (10ug) was co-transfected with empty pCDNA vector or

25 pCDNA containing the full-length T-bet cDNA, c-Maf, NFATp or p65 (10ug). The constructs also include the -400 to -40 IL-2 and IL-4 promoter luciferase reporters.

The Th0 mouse T cell thymoma EL4, which produces IL-2 and IL-4 but not IFN- γ , was transfected with a T-bet cDNA expression plasmid and the IFN- γ -luciferase reporter (Figure 5). Introduction of the T-bet expression plasmid resulted in

30 (approximately 20-30 fold) transactivation of the IFN- γ gene compared to empty vector

alone. This was in contrast to the absence of transactivation by two other factors, the Th2-specific transcription factor c-Maf and the Th non-selective transcription factor NFAT. Interestingly, although the NF κ B family member, p65, did not transactivate the IFN- γ reporter on its own, cotransfection of T-bet and p65 resulted in a synergistic activation.

5 Examination of the IL-2 promoter was also made using a region of the promoter known to be Th1-specific (Lederer, J.A., et al. 1994. *J. Immunol.* 152, 77-86). T-bet repressed the activity of the IL-2 promoter approximately 10 fold. This was especially apparent upon activation of the promoter by PMA and ionomycin. As before, 10 substantial transactivation of the IFN- γ gene was noted. T-bet activity was specific for the IL-2 and IFN- γ genes since no effect on transactivation of an IL-4 promoter (Figure 5) or a TNF- α promoter was present. These data demonstrate that T-bet specifically activates the transcription of the IFN- γ gene, and represses the transcription of the IL-2 gene.

15 To examine endogenous gene expression, EL4 cells were transiently transfected with T-bet or empty vector, and IFN- γ production measured by ELISA 48 hours after stimulation with PMA/ionomycin (Figure 5). Consistent with the transactivation data shown above, ectopic expression of T-bet in EL4 cells led to measurable IFN- γ production while transfection with vector control did not result in detectable IFN- γ .

20 **Example 7. Retroviral gene mediated transfer of T-bet into primary Th cells results in increased IFN- γ production**

The experiments described above argue strongly for a critical role of T-bet in controlling the transcription of the IFN- γ gene.

25 A bovine collagen-specific Th0 hybrid was transduced with retroviral constructs containing T-bet GFP or GFP only under the control of the TcR inducible IL-2 promoter. Transduced populations were FACS sorted on GFP twice, rested and then stimulated with anti-CD3 and supernatants collected at 60 hours to measure cytokine production by ELISA. (Figure 6). Control retroviral vectors which had no effect 30 included anti-sense T-bet.

To further test whether T-bet is responsible for the tissue-specific expression of IFN- γ , retroviral gene mediated transfer of T-bet into primary T cells, both non-transgenic and TcR transgenic, was performed. Two different bicistronic retroviruses expressing both T-bet and GFP were used. The first expresses T-bet under the control of 5 an IL-2 inducible promoter, and the second expresses T-bet under control of an MSCV LTR. Similar results were obtained with both constructs.

BALB/c CD4 T cells were infected after 36 hours of primary activation by anti-CD3 plus anti-CD28, harvested on day 7 and intracellular IFN-gamma and IL-2 staining performed 5 hours after stimulation with PMA and ionomycin as described in 10 Experimental Procedures. Data are shown as two-color plots showing GFP expression (FL1) versus intracellular cytokine (FL2) of events gated on expression of CD4. Primary T cells from MBP TcR transgenic mice were stimulated using MBP (Ac1-11) at 6 uM and infection performed on day 1 with IL-2/GFP and IL-2/T-bet/GFP. On day 7, 15 cells were sorted for GFP expression, rested for 1 day and then intracellular cytokine analysis performed after a 5 hour stimulation with PMA and ionomycin.

Naive MBP-transgenic or non-transgenic BALB/c CD4 T cells were activated with MBP 1-11 and anti-CD3 under non-polarizing conditions and were infected with retrovirus on day 1 after primary activation as described (Ouyang, W., et al. 1998. Immunity 9:745-755.). Cells were cultured for 7 days and then GFP expression 20 measured to determine percentage of cells infected. GFP positive cells were sorted and cytokine production measured by intracellular staining after an additional 4 hours stimulation with PMA plus ionomycin.

Transduction of both MBP-TcR transgenic and non-transgenic T cells with T-bet resulted both in an impressive increase in the number of cells producing IFN- γ and in the 25 amount of IFN- γ produced per cell as compared to cells transduced with GFP alone. (Figure 7).

Naive Thp cells, early after stimulation, produce large amounts of IL-2, which is then gradually replaced in polarized Th cells by the effector cytokines IFN- γ and IL-4. Polarized Th1 cells do continue to produce IL-2 but at amounts considerably less than 30 naïve Thp. Polarized Th2 cells shut off the production of IL-2. T-bet transduced Th cells

produced somewhat less IL-2 than GFP/RV control transduced cells, consistent with the repression of IL-2 promoter transactivation by T-bet that we observed in EL4 cells. The repression of IL-2 by T-bet is consistent with a function for T-bet in driving lineage commitment from a naive precursor cell into a fully differentiated effector cell.

5

Example 8. T-bet activates IFN- γ and represses IL-4 production in developing Th2 cells

The experiments above demonstrate that T-bet can direct unskewed Th cells into the Th1 pathway. The T-bet could force Th cells to direct their genetic program along a Th1 pathway even in the presence of stimuli that would ordinarily drive them into the Th2 pathway was tested. In the experiments in Figure 8, BALB/c CD4+ T cells were activated with anti-CD3 and anti-CD28 in the presence of rIL-4 and antibodies to IFN- γ and IL-12, retroviral infection performed at 36 hours, cells expanded with IL-2, GFP positive cells sorted on day 7 and cytokine production measured by intracellular staining after an additional 4 hours stimulation with PMA plus ionomycin. Transduction with GFP-RV alone resulted in a population that contained 13.4 % IL-4-producing cells and 0.9% IFN- γ producers (Figure 8). As expected, the Thp cells are not yet fully polarized at this time. Introduction of T-bet/GFP/RV produced a substantial shift of Thp into the Th1 pathway as evidenced by the large number of cells (50%) producing IFN- γ and the reduced number of cells producing IL-4 (3.5%), even under conditions (rIL-4 and anti-IL-12) that inhibit Th1 differentiation. Thus, T-bet can overcome the Th2-promoting signals delivered by cytokines to drive developing Th cells into the Th1 pathway.

Example 9. T-bet redirects polarized Th2 cells into the Th1 pathway

25 It has been demonstrated that reversibility of Th1 and Th2 populations is lost after long-term stimulation under polarizing conditions. Reversibility is largely abrogated after one week and is completely lost after 3 weeks (Murphy, E., et al. 1996. *J. Exp. Med.* 183, 901-913.). To determine whether T-bet could redirect the commitment of a pure population of already polarized Th2 cells, CD4+ T cells were cultured as 30 above and retroviral gene transduction performed at day 9 of culture. In Th cells

cultured for 9 days under Th2 polarizing conditions, control GFP/RV-transduced cells are virtually all IL-4 and IL-5 producers (23% and 11%) with barely detectable IFN- γ producer cells (6%) (Figure 9). Thus, as expected, almost complete polarization had occurred. Remarkably, introduction of T-bet into these fully polarized Th2 cells

5 redirected or converted them into polarized Th1 cells as evidenced both by the induction of IFN- γ expression and the loss of IL-4 and IL-5 expression. This conversion occurred in the presence of exogenous IL-4. Fully 77 % of T-bet-transduced Th2 cells now produced IFN- γ while the percentage of cells producing IL-4 and IL-5 has been reduced to 13 % and 1% respectively. These T-bet-transduced cells are therefore not Th0 cells

10 that produce both IFN- γ and IL-4. Therefore, T-bet has not simply induced IFN- γ expression in Th2 cells but has actually reprogrammed Th2 cells into the opposing Th1 subset.

Example 10. T-bet also redirects polarized Tc2 cells into the Th1 pathway

15 Although most attention has focused on the CD4+ T lymphocyte, it is apparent that cytotoxic CD8+ T cells also may also be divided into IFN- γ -producing (Tc1) and IL-4-producing (Tc2) subsets. The ability of T-bet to redirect fully polarized Tc2 cells into a Tc1 pathway was tested. Purified CD8+ T cells were therefore differentiated in culture under Tc2 polarizing conditions for 9 days to accomplish full differentiation.

20 Figure 10 demonstrates that T-bet transduced Tc2 cells, similar to T-bet transduced CD4 Th2 cells have been reprogrammed to produce IFN- γ (85% versus 15%) and to repress the production of IL-4 and IL-5 (3% versus 34% and 1% versus 45% respectively). Thus, T-bet can convert fully differentiated CD8+ Tc2 cells to Tc1 cells.

25 **Example 11. T-Bet is tyrosine phosphorylated**

To determine whether T-bet is a tyrosine phosphorylated protein, whole cell lysates from AE7 Th1 cells were prepared after incubation for 0, 5, 10, 30 minutes with pervanadate. Lysates were immunoprecipitated with anti-T-bet antiserum, separated by SDS-PAGE (8% gel), transferred to nitrocellulose, and probed with an anti-phosphotyrosine mAB 4G10. Following exposure, blots were stripped and reprobed

with anti-T-bet antisera. As shown in Figure 11, T-bet is clearly a tyrosine phosphorylated protein in T cells.

Example 12. Creation of a dominant negative T-bet molecule

5 Chimeric cDNA molecules were made with the T-bet DNA binding domain (residues 138-327) and the repressor domain of the *Drosophila* protein engrailed. The engrailed protein is a powerful, active repressor of transcription (Taylor, D., 1996. *Genes Dev.* 10, 2732; Li, J., Thurm, H., et al. 1997. *Proc. Natl. Acad. Sci. USA* 94, 10885). The T-bet-*engrailed* construct in vitro using a multimerized T-box consensus 10 site/TK minimal promoter luciferase reporter construct. As shown in Figure 12, T-bet/*engrailed* specifically and significantly represses the ability of wt T-bet to transactivate a T-box reporter construct at a 5:1 ratio, and does not repress transactivation of an NFAT or NF κ B reporter by NFATp and p65 expression constructs respectively.

15

Example 13. Mutations of the T box of the IL-2 promoter decrease IL-2 promoter activity

Recently, the crystal structure of the T-box region of the *Brachury* gene bound to DNA was solved and the amino acid moieties essential for specific DNA contacts or 20 for minor contacts deduced. Examination of the human and murine IL-2 proximal promoter shows that the critical nucleotides for binding a T-box family member are present. Specifically -240 to -220 bp of the murine IL-2 promoter has strong similarity to the consensus T-box site. The consensus T-box site is

AATTTTC**CACCTAGGTTGTG Δ AATT. The human IL-2 promoter comprises:**

25 gAgcTatCACCTAaGTGTGggcTa. The murine IL-2 promoter comprises: AAacTgcCACCTAaGTGTGggcTa. The mutated T-box mIL-2 promoter comprises: AAacTgctgtCTAacaIGggcTa. (DNA contacts are in bold, minor contacts are underlined). Transversional nucleotide substitutions shown by crystal structure to be essential for DNA-protein interactions were made within this putative T-box site in the 30 context of the murine -440 to -40 bp IL-2 promoter. The basal level (open bars) and the PMA (50ng/ml) plus ionomycin (1 μ M) induced (closed bars) promoter activity in Jurkat

cells (left) or AE7 Th1 clone (right) of IL-2 luciferase reporter constructs is shown (Figure 13).

The role of T-bet is to drive differentiation of Th cells as evidenced by its ability to simultaneously induce the IFN- γ gene and repress the IL-2 gene. The antigen-5 inexperienced Thp cell produces only IL-2. Upon stimulation, IL-2 production declines and is replaced by production of the Th mature effector cytokines. In particular, Th2 cells cease to make IL-2 as they acquire the ability to make IL-4, and the function of T-bet in simultaneously inducing IFN- γ and repressing IL-2 was especially obvious in Th2 cells. The ability of T-bet to simultaneously transactivate the IFN- γ promoter and 10 repress the IL-2 promoter is therefore consistent with a role for T-bet in propelling differentiation of the naïve Thp. T-bet has been shown to transactivate a construct containing only 3 kb of upstream promoter sequence, consistent with the presence of two T-box sites at positions -2300 to -2291 and -1957 to -1948. However, since this region of the promoter is not Th1-specific, it is likely that the T-box site in the third 15 intron is also important and there may well be additional T-box sites elsewhere in the gene.

The T-box domain has recently been co-crystallized with DNA and demonstrates a novel sequence-specific DNA recognition architecture in which the protein contacts DNA in both the major and minor grooves (Müller, C.W. and Herrmann, B.G. 1997. 20 Nature 389, 884-888). The consensus T box binding site as defined by target site selection in vitro is a palindrome 5'-GGGAATTCACACCTAGGTGTGAAATTCCC-3'. Inspection of the IL-2 promoter reveals an excellent T-box site at -240 to -220 just 5' of the NF κ B site to which recombinant T-bet protein binds. The binding of T-bet to the IL-2 promoter explains its isolation in the yeast one hybrid screen where the readout 25 depended simply on binding of T-bet to the T box site in the IL-2 promoter to drive an artificial reporter. Despite the clear repression of IL-2 promoter activity by T-bet, a decrease in IL-2 promoter activity upon mutation of the T-box site has been observed. However, that T-bet can still repress the transactivation of an IL-2 promoter construct in which that T-box site has been mutated. This suggests either the presence of another T- 30 box site in the IL-2 promoter, or interference with another positively acting factor that

binds close by. A good candidate for this factor is an activity described by Rothenberg and colleagues that binds to a site TGGGCC just adjacent to the T-box site (Chen, D. and Rothenberg, E.V. 1994. *J. Exp. Med.* 179, 931-942).

In addition, T-bet represses the Th2 program in Thp and Th2 cells. This is

5 unlikely to be the direct result of an imbalance between IFN- γ and IL-4 in favor of the former. The effect of T-bet in repressing the Th2 program while simultaneously enhancing the Th1 program is reminiscent of GATA-3 and c-Maf, both of which indirectly repress IFN- γ expression, the former through influencing expression of the IL-12 receptor β 2 chain (Ho, I-C., et al. 1998. *J. Exp. Med.* 188:1859-1866.; Ouyang, 10 W., et al. 1998. Inhibition of Th1 developmental mediated by GATA-3 through an IL-4 independent mechanism. *Immunity* 9:745-755). However, unlike GATA-3 and c-Maf, T-bet can actually convert fully polarized effector Th cells into the opposing pathway.

Equivalents

15 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a T-bet protein.
5
2. The nucleic acid molecule of claim 1, which comprises the nucleotide sequence shown in SEQ ID NO:1.
3. The nucleic acid molecule of claim 2, which comprises the nucleotide sequence of SEQ ID NO: 3.
10
4. The nucleic acid molecule of claim 1, which has at least 70% nucleotide identity with at least about 700 contiguous nucleotides of SEQ ID NO:1.
15
5. The nucleic acid molecule of claim 1, which has at least 70% nucleotide identity with at least about 500 contiguous nucleotides of SEQ ID NO:3.
20
6. The nucleic acid molecule of claim 1, which has at least 90% nucleotide identity with at least about 700 contiguous nucleotides of SEQ ID NO:1.
25
7. The nucleic acid molecule of claim 1, which has at least 90% nucleotide identity with at least about 500 contiguous nucleotides of SEQ ID NO:3.
8. A vector comprising the nucleic acid molecule of claim 1.
9. The vector of claim 8, which is an expression vector.
10. A host cell containing the vector of claim 9.
30
11. A method for producing a T-bet protein comprising culturing the host cell of claim 10 in a suitable medium until a T-bet protein is produced.

12. The method of claim 11, further comprising isolating the T-bet protein from the medium or the host cell.

5 13. An isolated T-bet protein.

14. The protein of claim 12, which comprises the amino acid sequence encoded by the nucleic acid sequence of SEQ ID NO:1.

10 15. The protein of claim 12, which comprises the amino acid sequence encoded by the nucleic acid sequence of SEQ ID NO:3.

16. The protein of claim 12, which comprises the amino acid sequence of SEQ ID NO: 2.

15

17. The protein of claim 12, which comprises the amino acid sequence of SEQ ID NO: 4.

18. The protein of claim 12, which has at least 70% amino acid identity with 20 the protein shown in SEQ ID NO:2.

19. The protein of claim 12, which has at least 70% amino acid identity with the protein shown in SEQ ID NO:4.

25 20. The protein of claim 12, which has at least 90% amino acid identity with the protein shown in SEQ ID NO:2 and having the ability to bind to DNA.

21. A fusion protein comprising a T-bet protein operatively linked to a polypeptide other than T-bet.

30

22. Antibodies that specifically bind T-bet protein.

23. The antibodies of claim 21, which are polyclonal antibodies.
24. The antibodies of claim 21, which are monoclonal antibodies.
- 5 25. The antibodies of claim 21, which are coupled to a detectable substance.
26. A nonhuman transgenic animal that contains cells carrying a transgene
encoding a T-bet protein.
- 10 27. A method for detecting the presence of T-bet in a biological sample
comprising contacting the biological sample with an agent capable of detecting an
indicator of T-bet activity such that the presence of T-bet is detected in the biological
sample.
- 15 28. A method for modulating T-bet activity in a cell comprising contacting
the cell with an agent that modulates T-bet activity such that T-bet activity in the cell is
modulated.
- 20 29. A method for identifying a compound that modulates the activity of a T-
bet protein, comprising
 - providing an indicator composition that comprises a T-bet protein;
 - contacting the indicator composition with a test compound; and
 - determining the effect of the test compound on the activity of the T-bet protein in
- 25 the indicator composition to thereby identify a compound that modulates the activity of
a T-bet protein.

30. The method of claim 27, wherein:

the indicator composition comprises a T-bet protein and a DNA molecule to which the T-bet protein binds; and

the effect of the test compound on the activity of the T-bet protein is determined

5 by evaluating the binding of the T-bet protein to the DNA molecule in the presence and absence of the test compound.

31. The method of claim 27, wherein:

the indicator composition is a cell comprising a T-bet protein and a reporter gene

10 responsive to the T-bet protein; and

the effect of the test compound on the activity of the T-bet protein is determined by evaluating the expression of the reporter gene in the presence and absence of the test compound.

15 32. The method of claim 27, further comprising determining the effect of the test compound on an immune response to thereby identify a compound that modulates an immune response.

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HUMAN AND MOUSE T-BET ALIGNMENT

	10	20	30	40	50	60
HUMAN	MGIVEPGCGDMLTGTEPM	PGSDEGRAPGADPQHRYF	YPEPGAQD	ADE	RGGGSLGSPY	PG
MOUSE	MGIVEPGCGDMLTGTEPM	P-SDEGRGP	GADQHRF	YFPEPGAQD	OPTDRRA	GSSLGTP
	10	20	30	40	50	
CONS	MGIVEPGCGDMLTGTEPM	P-SDEGR	PGAD QHR	YFPEPGAQD	RR G	SLG PY G
	70	80	90	100	110	120
HUMAN	GALVPA	PSSREL	GAYA	YPPR	QAGFP	GAGESF
MOUSE	GALVPA	PAPFR	FLGSF	AYPPR	QAGFP	GAGESF
	60	70	80	90	100	110
CONS	GALVPA	P	RFLG	AYPPR	Q	AGFP
	130	140	150	160	170	180
HUMAN	PREDYALPAGL	EVSGKL	RVAL	NHH	LLWS	KFNQHQTE
MOUSE	PREDYALPAGL	EVSGKL	RVAL	NHLL	WSKFNQHQTE	MIITKQGRM
	120	130	140	150	160	170
CONS	PREDYALPAGL	EVSGKL	RVAL	NHLL	WSKFNQHQTE	MIITKQGRM
	190	200	210	220	230	240
HUMAN	SHYRMFV	DVVLV	DQHHWRY	QSGK	WVQCGK	AE
MOUSE	SHYRMFV	DVVLV	DQHHWRY	QSGK	WVQCGK	AE
	180	190	200	210	220	230
CONS	SHYRMFV	DVVLV	DQHHWRY	QSGK	WVQCGK	AE
	250	260	270	280	290	300
HUMAN	KLKL	TNNKGASNN	VTQMI	VLSL	HKYQ	PRLH
MOUSE	KLKL	TNNKGASNN	VTQMI	VLSL	HKYQ	PRLH
	240	250	260	270	280	290
CONS	KLKL	TNNKGASNN	VTQMI	VLSL	HKYQ	PRLH

FIGURE 1A

	310	320	330	340	350	360
HUMAN	AVTAYQNAEITQLKIDNNPFAKGFR <u>ENFESMYTSVDT</u> SIPSPPGPNCQFLGGDHYSPLLP					
MOUSE	AVTAYQNAEITQLKIDNNPFAKGFR <u>ENFESMYASVDT</u> SVPSPPGPNCQLLGGDPFSPLLS					
	300	310	320	330	340	350

CONS AVTAYQNAEITQLKIDNNPFAKGFRENFESMY SVDTS PSPPGPNCQ LGGD FSPLL

	370	380	390	400	410	420
HUMAN	NQYPVPSRFYDLPQAKDVVVQAYWLGA <u>PRDH</u> SYAEFRAVSMKPAFLPSAPGPTMSYY					
MOUSE	NQYPVPSRFYDLPQPKDMISQPYWLGTREH <u>SYAEFRAVSMKPTLL</u> PSAPGPTVPPY					
	360	370	380	390	400	410

CONS NQYPVPSRFYDLPQK KD Q YLWG PR HSYEAEFRAVSMKP LPSAPGPT YY

	430	440	450	460	470	480
HUMAN	RGQEVLAPGAGWPVAPQYPPKMGPA <u>SWFRPMRTLPM</u> EPGP <u>GGSEGRG</u> PEDQGPPLVWTEI					
MOUSE	RGQDVLAPGAGWPVAPQYPPKMS <u>PAGWFRPMRTLPM</u> DPGLGSSE <u>QG</u> ---SSPSLWPEV					
	420	430	440	450	460	470

CONS RGQ VLAPGAGWPVAPQYPPKM PA WFRPMRTLPM PG G SE G P W E

	490	500	510	520	530	
HUMAN	APIRPESSD <u>SGL</u> GEGDSKRRRVSPY <u>PSSGD</u> SSSPAGAPS <u>PF</u> DKE <u>AE</u> G <u>QFYNY</u> FPN					
MOUSE	TSLOPE <u>PSD</u> SGL <u>GEGD</u> TKRRRISPY <u>PSSG</u> DSSSPAGAPS <u>PF</u> D <u>KETE</u> G <u>QFYNY</u> FPN					
	480	490	↑↑↑ 500	510	520	530

CONS PE SDSGLGEGD KRRR SPYPSSGDSSSPAGAPSPFDKETEGQFYNYFPN

FIGURE 1A (continued)

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HUMAN AND MOUSE T-BET NUCLEOTIDE SEQUENCE ALIGNMENT

	10	20	30	40	50	60
HUMAN	ATGGGCATCGTGGACCGGGTTGGAGACATGCTGACCGGACCGAGCCGATGCCGGG					
MOUSE	ATGGGCATCGTGGACCGGGCTGCGGAGACATGCTGACCGGACCGAGCCGATGCCG	10	20	30	40	50
HUMAN	AGCGACGAGGGCGGGCGCTGGCGCACCCGAGCAGCACCGCTACTCTACCGGAGCG	70	80	90	100	110
MOUSE	AGTGAACGAGGGCGGGGGCGGAGCGACCAACAGCATCGTTCTATCCGAGCG	60	70	80	90	100
HUMAN	GGCGCGCAGGACCGCGACGAGCGTCGCGGGGCGCAGCCTGGGTCTCCCTACCGGG	130	140	150	160	170
MOUSE	GGCGCACAGGACCCACCGATCGCCGAGCAGTAGCAGCCTGGGGAGCGCCACTCTGG	120	130	140	150	160
HUMAN	GGCGCTTGGTGCCTGGCCGGCCGCGAGCGGCTTCTGGAGCCTACCGCTACCGCGGGA	190	200	210	220	230
MOUSE	GGCCCTTGGTGCCTGGCCGGCCGGTCCCTGGATCCTCGCCATCCGGCTACCGCCCCGG	180	190	200	210	220
HUMAN	CCCCAGCGCGGGCTTCCCGCGCGGGAGCTCTCCGGCGACGCCGCGGAGCGCCAG	250	260	270	280	290
MOUSE	GCTCAGGGCTGGCTGGCTTCCGGGCTGGAGTTCTCCGGCGGGTCTACCGGGAGCGGG	240	250	260	270	280
HUMAN	GGCTACCGCCGGGAGGGCTACGCCCGGAGCCGGCGCCGGCTACCGGGGG	310	320	330	340	350
MOUSE	GGCTACCGCCGGTGGATGGCTACCTGGCCCTGACCGCGGGGGCTACCGGG	300	310	320	330	340
HUMAN	CCGGCTGAGGACTACGGCTACCCGGGACTGGAGGTGTCGGGAAACTGAGGGTCGG	370	380	390	400	410
MOUSE	CCGGCGAGGACTACGGCATTCGGCCGGGGTTGGAGGTGTCGGGAAGCTGAGAGTCGG	360	370	380	390	400

FIGURE 1B

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	430	440	450	460	470	480
HUMAN	CTCAACACCCACCTGTTGTCAGTTAACGACCCAGACAGAGATGATCATCACC					
MOUSE	CTCAGCAACACCCACCTGTTGTCAGTTAACGACCCAGACAGAGATGATCATCACC					
	420	430	440	450	460	470
	490	500	510	520	530	540
HUMAN	AAGCAGGGACGGCGGATGTTCCATTCTGTCAATTACTGTGGCCGGCTGGAGCCCAC					
MOUSE	AAGCAAGGACGGGAATGTTCCATTCTGTCTTCACCGTGGCCGGCTGGAGCCCAC					
	480	490	500	510	520	530
	550	560	570	580	590	600
HUMAN	AGCCACTACAGGATGTTGTCAGTGGCTTGGTGGACAGCACCCTGGCGGTACCG					
MOUSE	AGCCATTACAGGATGTTGTCAGTGGCTTGGTGGACAGCACCCTGGCGGTACCG					
	540	550	560	570	580	590
	610	620	630	640	650	660
HUMAN	AGCGGAAGTGGGTGCACTGGTGGAAAGGCCAGGGCAGCATGCCAGGAACCGCTGTAC					
MOUSE	AGCGGAAGTGGGTGCACTGGTGGAAAGGCCAGGAAGGCAGCATGCCAGGAACCGCTTAT					
	600	610	620	630	640	650
	670	680	690	700	710	720
HUMAN	GTCCACCCGGACTCCCCAACACAGGAGCGCAGTGGATGCCAGGAAGTTCAATTGGG					
MOUSE	GTCCACCCAGACTCCCCAACACCGGAGGCCACTGGATGCCAGGAAGTTCAATTGGG					
	660	670	680	690	700	710
	730	740	750	760	770	780
HUMAN	AAACTAAAGCTCACAAACACAAAGGGGGCTCCAACAAATGTGACCCAGATGATTGGCTC					
MOUSE	AAGCTAAAGCTACCAACAAACAAAGGGGGCTCCAACAAATGTGACCCAGATGATCGCTCG					
	720	730	740	750	760	770
	790	800	810	820	830	840
HUMAN	CAGTCCCTCCATAAAGTACCAAGCCCCGGCTGCATATCGTTGAGGTGAACGACGGAGGCCA					
MOUSE	CAGTCTCCACAAAGTACCAAGCCCCGGCTGCACATGTGGAGGTGAATGAGGAGCCA					
	780	790	800	810	820	830

FIGURE 1B (continued)

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	850	860	870	880	890	900
HUMAN	GAGGCAGCTGCAACGCTTCAACACGCAATCTTACTTCCAAGAAACCCAGTCATT					
MOUSE	GAGGCCTGCCAGTGCTCTAACACACAGCTTACTTCCAAGAGACCCAGTCATT					
	840	850	860	870	880	890
	910	920	930	940	950	960
HUMAN	GCCGTGACTGCCCTACCAGAATGCCGAGATTACTCAGCTGAAAATTGATAATAACCCCTT					
MOUSE	GCAGTGACTGCCCTACCAGAAGCAGAGATCACTCAGCTGAAAATCGACACACACCCCTT					
	900	910	920	930	940	950
	970	980	990	1000	1010	1020
HUMAN	GCCAAAGGATTCGGGAGAACCTTGAGTCATGTACACATCTTGCACCCAGCATCCCC					
MOUSE	GCCAAAGGATTCGGGAGAACCTTGAGTCATGTACCGCATCTGATACGAGTGTCCCC					
	960	970	980	990	1000	1010
	1030	1040	1050	1060	1070	1080
HUMAN	TCCCGCCTGGACCCAACTGTCAATTCTTGGGGAGACTACTACTCTCTCTACCC					
MOUSE	TCGCAACCTGGACCCAACTGTCAACTGCTTGGGGAGACCCCTTCACCTTCTATCC					
	1020	1030	1040	1050	1060	1070
	1090	1100	1110	1120	1130	1140
HUMAN	AACCAGTATCCGTCCCCAGCGCTTCTACCCGACCTTCTGGCCAGGGGAAGGATGTG					
MOUSE	AACCAGTATCCGTCCCCAGCGCTTCTACCCGACCTTCCAGGCCAGGGATATG					
	1080	1090	1100	1110	1120	1130
	1150	1160	1170	1180	1190	1200
HUMAN	GTTCCCCAGGCTTACTGGCTGGGGCCCCGGGACACAGCTATGAGGCTGAGTTCGA					
MOUSE	ATCTCACAGCCTTACTGGCTGGGACACCTCGGGAACAGTTATGAAGCGGAGTCCGA					
	1140	1150	1160	1170	1180	1190
	1210	1220	1230	1240	1250	1260
HUMAN	GCAGTCAGCATGAAGCCCTGCAATTCTTGCCTCTGCCCTGGGCCACATGTCTACTAC					
MOUSE	GCTCTGAGCATGAAGCCACACTCTACCCCTGCCCCGGGGCACTGTGCCCTACTAC					
	1200	1210	1220	1230	1240	1250

FIGURE 1B (continued)

HUMAN	CGAGGCCAGGAGTCTGGCACCTGGAGCTGGCTGCCCTGGCACCCAGTACCCCTCC	1270	1280	1290	1300	1310	1320
MOUSE	CGGGGCAAGACGTCTGGGCCCTGGAGCTGGCTGCCGTGCCCCCTCAATACCCGCC	1260	1270	1280	1290	1300	1310

HUMAN	AAGATGGGCCGGCAGCTGGTCCGCCCTATGCGACTCTGCCATGGAACCCGGCC	1330	1340	1350	1360	1370	1380
MOUSE	AAGATGAGGCCAGCTGGCTGGTCCGCCATCGAACCTCTGCCATGGACCCGGCC	1320	1330	1340	1350	1360	1370

HUMAN	GGAGGCTCAGAGGGACGGGACAGAGGACCCAGGGTCCCCCTGGTGTGACTGAGATT	1390	1400	1410	1420	1430	1440
MOUSE	GGATCCTCAGAGGAACAGGGCTCCT-----CCCCCTGGCTGTGGCTGGAGTC	1380	1390	1400	1410	1420	

HUMAN	GCCCCATCCGGCGGAATTCACTGATTCAAGGACTGGCGAAGGAGACTCTAAGAGGAGG						
MOUSE	ACCTCCCTCAGCCGAGCCAGCGACTCAGGACTAGGCAGAGACACTAAGAGGAGG	1430	1440	1450	1460	1470	1480

HUMAN	CGCGTGTCCCCCTATCCCTCAGTGGTGACAGCTCTCCCTGCTGGGCCCTTCCT	1510	1520	1530	1540	1550	1560
MOUSE	AGGATATCCCCATCCCTCAGTGGGACAGCTCTCCCTGCTGGGCCCTTCCT	1490	1500	1510	1520	1530	1540

HUMAN	TTTGATAAGGAAGCTGAAGGACAGTTTATACTTATTTCCAACTGA	1570	1580	1590	1600		
MOUSE	TTTGATAAGGAACCGAAGGCCAGTTTATAATTATTTCCAACTGA	1550	1560	1570	1580	1590	

FIGURE 1B (continued)

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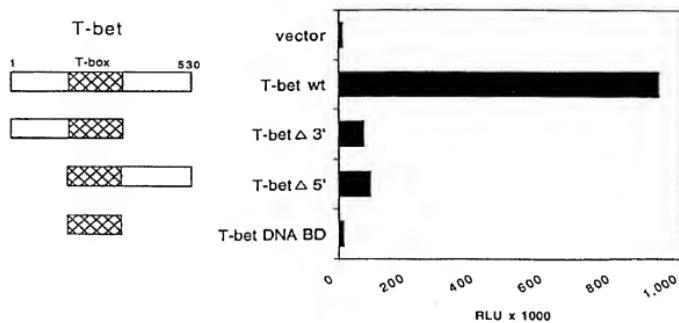
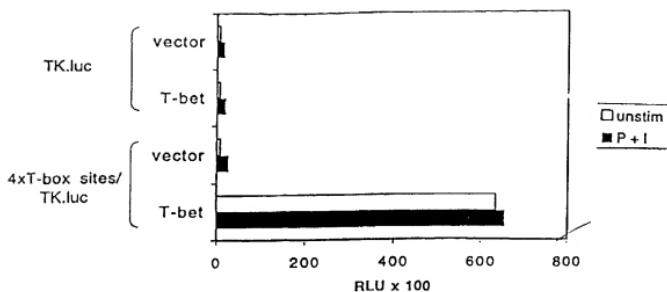


FIGURE 2

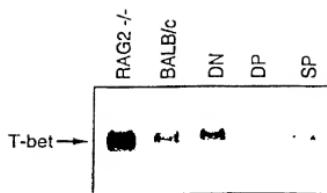


FIGURE 3A

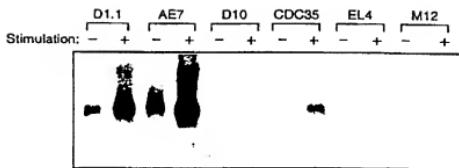


FIGURE 3B

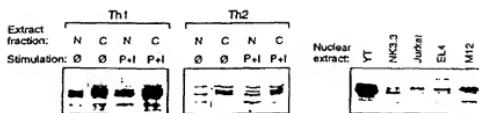


FIGURE 3C

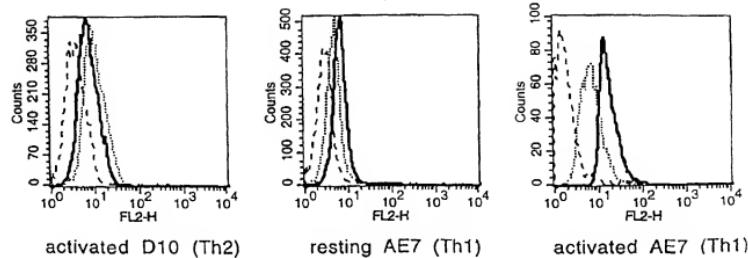


FIGURE 3D

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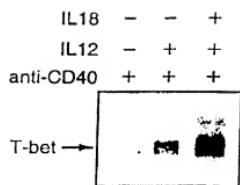


FIGURE 4A

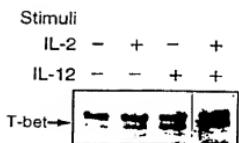


FIGURE 4B

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T-bet Transactivates the IFN- γ Gene in Th Cells

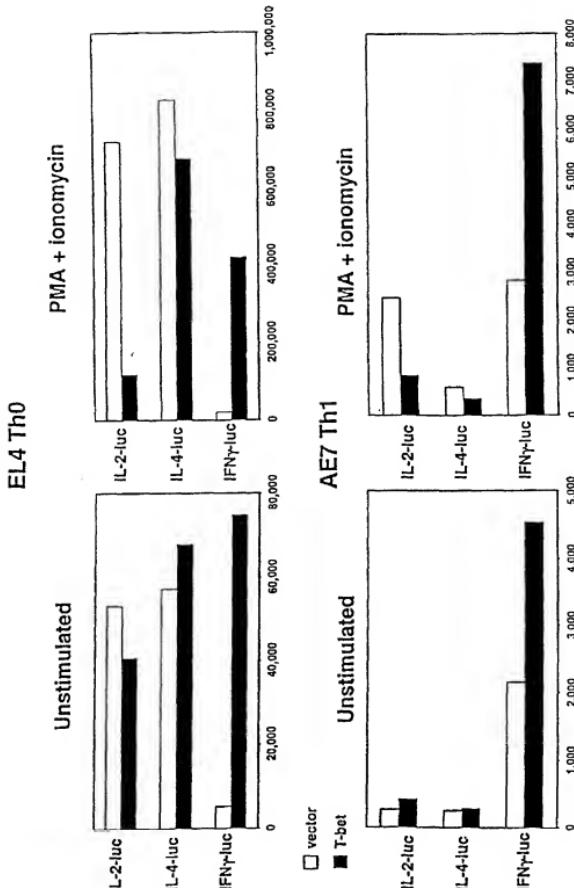


FIGURE 5

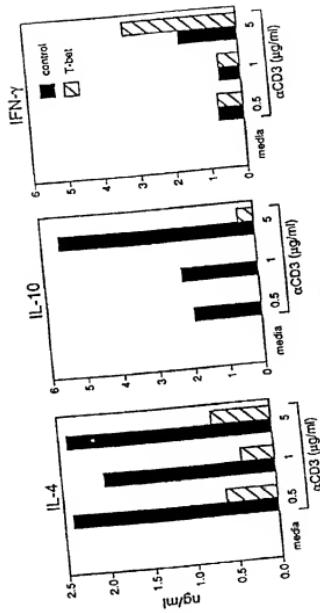


FIGURE 6

T-bet Strongly Induces $\text{IFN-}\gamma$ and modestly Represses
 IL-2 in Primary T Cells

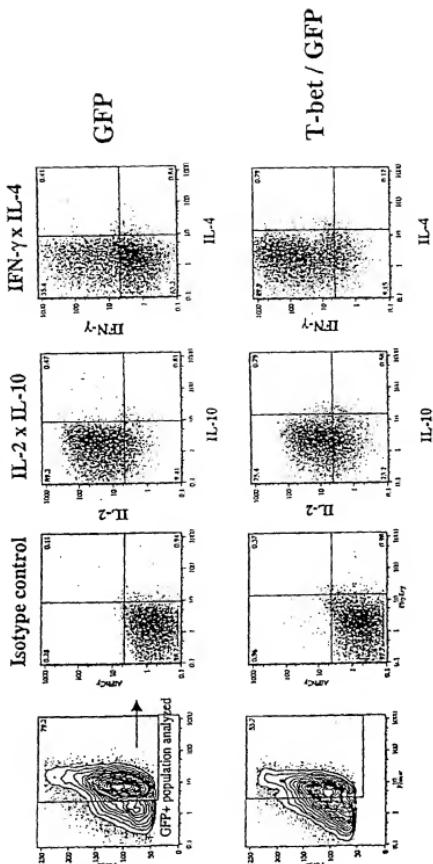
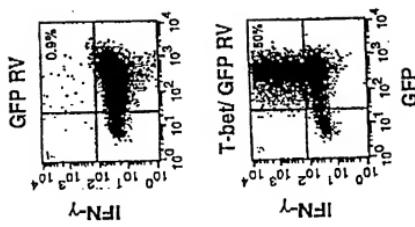


FIGURE 7

FIGURE 8A

**T-bet Induces IFN- γ Production
in Developing Th2 Cells**



T-bet Inhibits IL-4 Production
In Developing Th2 Cells

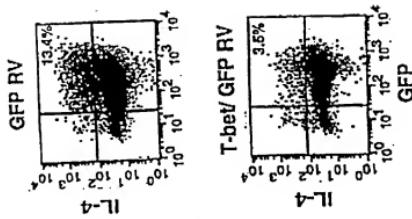


FIGURE 8B

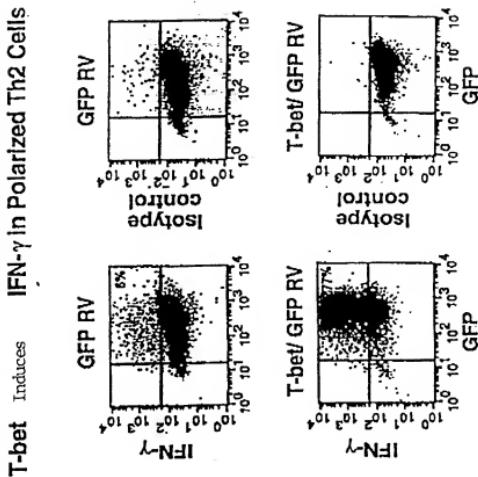


FIGURE 9A

T-bet Represses IL-2 In
Polarized Th2 Cells

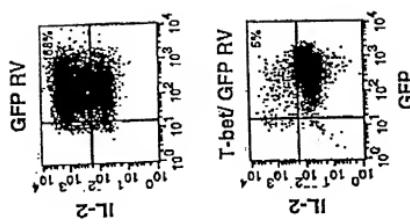


FIGURE 9B

T-bet Represses IL-4 in
Polarized Th2 Cells

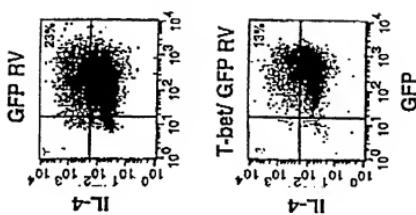


FIGURE 9C

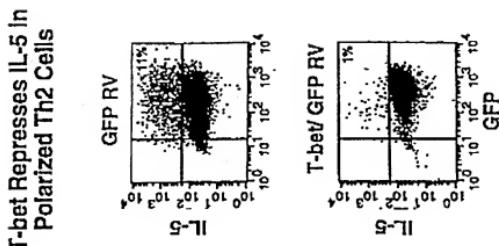


FIGURE 9D

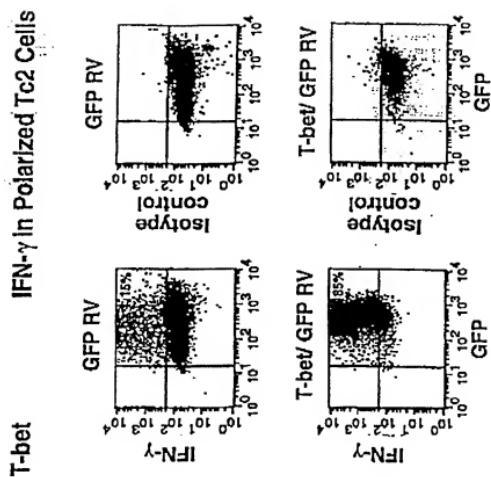


FIGURE 10A

T-bet Represses IL-2 In
Polarized Tc2 Cells

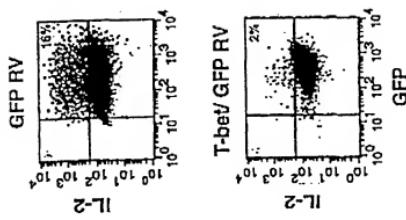


FIGURE 10B

T-bet Represses IL-5 in
Polarized Tc2 Cells

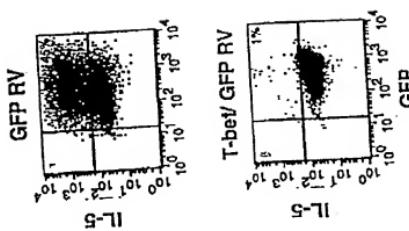


FIGURE 10C

T-bet Represses IL-4 in
Polarized Tc2 Cells

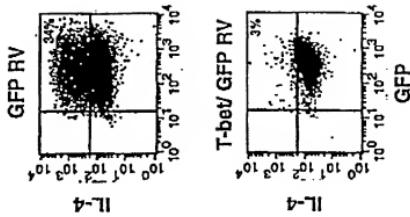


FIGURE 10D

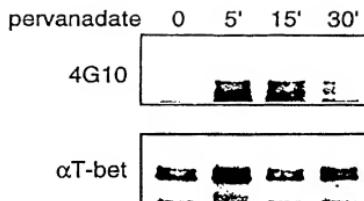


FIGURE 11

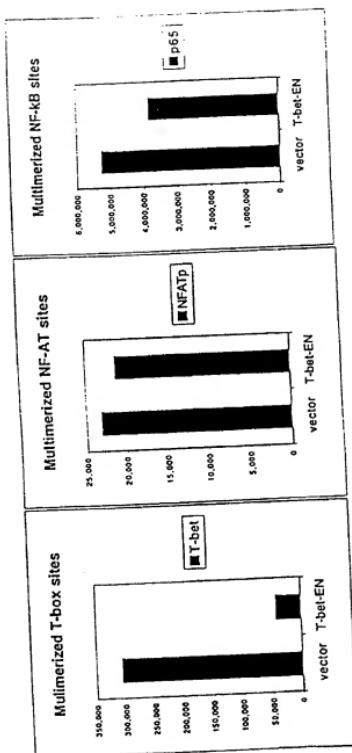


FIGURE 12

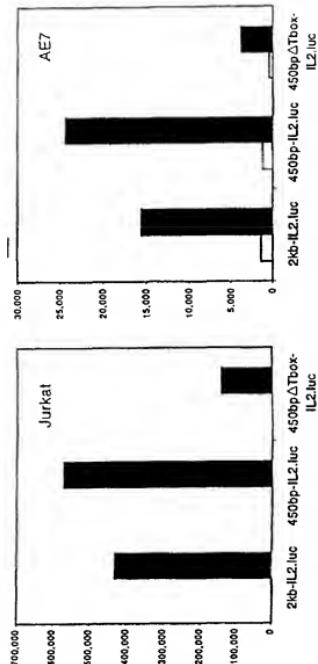


FIGURE 13

- 1 -

SEQUENCE LISTING

<110> President and Fellows of Harvard College

<120> T-BET COMPOSITIONS AND METHODS OF USE THEREOF

<130> HUI-040PC

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INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/US 00/15345

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C12N15/12 C12N5/10 C07K14/47 C07K16/18 G01N33/50
 C12Q1/68 A01K67/027 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHEDMinimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K G01N C12Q A01K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, STRAND, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	SZABO S.J. ET AL.: "A novel transcription factor, T-bet, directs Th1 lineage commitment." CELL, vol. 100, March 2000 (2000-03), pages 655-669, XP002148616 the whole document ---	1-32
P, X	DATABASE EMBL SEQUENCES 'Online! Accession No. AF093098, 16 October 1999 (1999-10-16) YANG S.: "Cloning and characterization of a new member of T-box gene Family (H. sapiens TBLYM)." XP002148618 TBLYM is identical to T-bet --- -/-	1-32

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other specific document as specified
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 2000	13/10/2000
Name and mailing address of the ISA European Patent Office, P. B. 5818 Patentlaan 2 NL-1025 AH Amsterdam Tel: (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Gall1, I

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/15345

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category ²	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BULFONE A ET AL: "T-brain-1: a homolog of Brachyury whose expression defines molecularly distinct domains within the cerebral cortex" NEURON, CAMBRIDGE, MA, US, vol. 15, no. 1, July 1995 (1995-07), pages 63-78, XP000864769 the whole document -----	1-32
A	SMITH J: "T-box genes: what they do and how they do it" TRENDS IN GENETICS, NL, ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, vol. 15, no. 4, April 1999 (1999-04), pages 154-158, XP004162854 ISSN: 0168-9525 the whole document -----	1-32
A	NOBLE A. ET AL.: "Regulation of Tc1/Tc2 and Th1/Th2 subset commitment by altered peptides and TcR-mediated signalling events." FASEB J., vol. 13, no. suppl., 15 March 1999 (1999-03-15), page A939 XP002148617 the whole document -----	1-32

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
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(54) Title: T-BET COMPOSITIONS AND METHODS OF USE THEREOF

(57) Abstract: Isolated nucleic acid molecules encoding T-bet, and isolated T-bet proteins, are provided. The invention further provides antisense nucleic acid molecules, recombinant expression vectors containing a nucleic acid molecule of the invention, host cells into which the expression vectors have been introduced and non-human transgenic animals carrying a T-bet transgene. The invention further provides T-bet fusion proteins and anti-T-bet antibodies. Methods of using the T-bet compositions of the invention are also disclosed, including methods for detecting T-bet activity in a biological sample, methods of modulating T-bet activity in a cell, and methods for identifying agents that modulate the activity of T-bet.

T-BET COMPOSITIONS AND METHODS OF USE THEREOF**Government Funding**

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Background of the Invention

10 Cells of the immune system alter patterns of gene expression in response to extracellular and intracellular signals. A group of polypeptides, designated cytokines or lymphokines, which affect a range of biological activities in several cell types, are among the most important of these signals. While many cell types in the immune system secrete cytokines, the T helper (Th) lymphocyte is the major source of these 15 polypeptides. More than a decade ago it was discovered that Th cells differentiate into two distinct subsets, Th1 and Th2, upon T cell receptor engagement, defined both by their distinct functional abilities and by unique cytokine profiles (Paul and Seder, 1994, Cell 76, 241-251; Mosmann and Coffman, 1989, Annu. Rev. Immunol. 7, 145-173; Mosmann et al., 1986, J. Immunol. 136, 2348-2357; Snapper and Paul, 1987, Science 20 236, 944-947). Th1 cells mediate delayed type hypersensitivity responses and macrophage activation while Th2 cells provide help to B cells and are critical in the allergic response (Mosmann and Coffman, 1989, Annu. Rev. Immunol. 7, 145-173; Paul and Seder, 1994, Cell 76, 241-251; Arthur and Mason, 1986, J. Exp. Med. 163, 774-786; Paliard et al., 1988, J. Immunol. 141, 849-855; Finkelman et al., 1988, J. Immunol. 141, 25 2335-2341). The evidence that Th1 cells directed cell-mediated immunity while Th2 cells contributed to humoral responses fit nicely with the observations that an organism tends to mount either a cell-mediated or humoral response, but not both, in response to pathogens. These functional differences between the Th subsets can be explained most easily by the activities of the cytokines themselves. IFN- γ is the "signature" cytokine of 30 Th1 cells although Th1 cells also produce IL-2, TNF and LT. The corresponding

“signature” cytokine for Th2 cells is IL-4. Th2 cells also secrete IL-5, IL-6, IL-9, IL-10 and IL-13.

Upon encountering antigen, the naive CD4+ T helper precursor (Thp) cell enacts a genetic program that ultimately sends it down a Th1 or Th2 lineage. While it is clear that polarization can be achieved by manipulating the antigen and costimulatory signals i.e. the “strength of signal” received by the Thp (Constant and Bottomly, 1997, *Annu. Rev. Immunol.* 15, 297-322), the most potent inducers of effector Th cells are undoubtedly the cytokines themselves. IL-4 promotes Th2 differentiation and simultaneously blocks Th1 development, an effect that is mediated via the Stat6 signaling pathway. Thus, mice that lack IL-4 or Stat6, fail to develop Th2 cells (Kopf et al., 1993, *Nature* 362, 245-248; Kuhn et al., 1991, *Science* 254, 707-710; Kaplan et al., 1996, *Immunity* 4, 313-319; Shimoda et al., 1996, *Nature* 380, 630-633; Takeda et al., 1996, *Nature* 380, 627-630). In contrast, IL-12, IL-18 and IFN- γ are the cytokines critical for the development of Th1 cells (Hsieh et al., 1993, *Science* 260, 547-549; Okamura et al., 1995, *nature* 378, 88-91; Gu et al., 1997, *Science* 275, 206-209; Meraz et al., 1996, *Cell* 84, 431-442; Magram et al., 1996, *Immunity* 4, 471-481). IFN- γ , acting via the Stat1 pathway (Meraz et al., 1996, *Cell* 84, 431-442), and IL-12, acting via the Stat-4 signaling pathway (Jacobson et al., 1995, *J. Exp. Med.* 181, 1755-1762) together promote the differentiation of Th1 cells and block commitment to the Th2 lineage (Szabo et al., 1995, *Immunity* 2, 665-675; Szabo et al., 1997, *J. Exp. Med.* 185: 817-824). Mice deficient in IL-12 or Stat4 do not have Th1 cells (Magram et al., 1996, *Immunity* 4, 471-481; Takeda et al., 1996, *Nature* 380, 627-630; Shimoda et al., 1996, *Nature* 380, 630-633). Another important Th1-inducing cytokine is IL-18, whose receptor is related to the IL-1 receptor family (Cerretti et al., 1992, *Science* 256, 97-100). Mice lacking IL-18 have defective in vivo Th1 responses (Takeda et al., 1998, *Immunity* 8, 383-390) and both IL-12 and IL-18 regulate IFN- γ expression (Barbulescu et al., 1998, *Eur. J. Immunol.* 27, 1098-1107; Robinson et al., 1997, *Immunity* 7, 571-581; Ahn et al., 1997, *J. Immunol.* 159, 2125-2131). The cytokines themselves, then, form a positive and negative feedback system that drives Th polarization (Powrie and Coffman, 1993, *Immunol. Today* 14, 270-274; Scott, 1991, *J. Immunol.* 147, 3149;

Maggi et al.. 1992. *J. Immunol.* 148, 2142; Parronchi et al., 1992. *J. Immunol.* 149, 2977; Fargeas et al.. 1992. *Eur. J. Immunol.* 149, 2977; Manetti et al., 1993. *J. Exp. Med.* 177, 1199; Trinchieri, 1993. *Immunol. Today* 14, 335-338; Macatonia et al., 1993, *Immunol.* 5, 1119; Seder et al.. 1993, *Proc. Natl. Acad. Sci. USA* 90, 10188-10192; Wu 5 et al., 1993. *J. Immunol.* 151, 1938; Hsieh et al., 1993. *Science* 260, 547-549) (reviewed in (Seder and Paul, 1994. In *Annual Review of Immunology*, Vol. 12, 635-673; Paul and Seder, 1994, *Cell* 76, 241-251; O'Garra, 1998, *Immunity* 8, 275-283).

Over the last few years. significant progress has been made in identifying the transcription factors that control the transition of a Thp to a Th2 cell as evidenced by the 10 capacity of such factors to drive IL-4 production reviewed in (Glimcher and Singh, 1999 *Cell* 96, 13-23; Szabo et al.., 1997, *Current Opinions in Immunology* 9, 776-781). The provision of three distinct proteins, the c-Maf proto-oncogene, the transcription factor Nuclear Factor of Activated T cells (NFAT), and a novel nuclear antigen, NFAT- 15 Interacting Protein 45 kD (NIP45), have been shown to confer on a non-T cell the ability to produce endogenous IL-4 (Hodge et al., 1996, *Science* 274, 1903-1905; Ho et al., 1998, *J. Exp. Med.* 188:1859-1866). These factors and others such as GATA-3 (Zheng and Flavell, 1997. *Cell* 89, 587-596) and Stat6 clearly can drive the production of IL-4, and therefore the development of Th2 cells, both *in vitro* and *in vivo*.

In contrast. little is known about the molecular basis of Th1 differentiation. For 20 example, the only known transcription factors whose absence results in a failure to generate Th1 cells are Stat4 (Thierfelder et al., 1996, *Nature* 382, 171-174; Kaplan et al., 1996, *Nature* 382, 174-177) and IRF-1(Lohoff et al., 1997, *Immunity* :681-689; Taki et al., 1997, *Immunity* 6:673-679), neither of which is Th1-specific. The Ets family member ERM which is induced by IL-12 in a Stat4-dependent manner has recently been 25 reported to be Th1-specific but it does not affect the production of Th1 cytokines (Ouyang et al., 1999. *Proc. Natl. Acad. Sci.* 96:3888). The absence of Th1 cells in Stat4 deficient mice is secondary to the failure of IL-12 to drive the Th1 program while the lack of Th1 cells in IRF-1 deficient mice is likely due to its direct effect in controlling transcription of the IL-12 gene (Lohoff et al., 1997, *Immunity* 6: 681-689; Taki et al., 30 1997, *Immunity* 6:673-679). However, some of the signaling pathways upstream of such putative Th1-specific regulatory factors are beginning to be elucidated. The p38 kinase

is one such signaling molecule as demonstrated by the ability of constitutively activated MAP kinase kinase 6 (MKK6) to boost IFN- γ production. Conversely, overexpression of a dominant negative p38 MAP kinase or targeted disruption of Jnk2 or Jnk1 reduces Th1 responses (Rincón et al., 1998, EMBO J. 17, 2817-2829; Yang et al., 1998, Immunity 9, 5 575-585; Dong et al., 1998, Science 282, 2092-2095). The JNK signaling pathway might affect Th development by a direct effect on the transcription of the IFN- γ gene, but this has not been shown. For example, the ATF-2 and AP-1 transcription factors are both substrates of JNK kinases and these factors as well as NF κ B and Stat4 proteins are known to bind to sites in the IFN- γ promoter (Zhang et al., 1998, Immunol. 161, 6105-10 6112; Ye et al., 1996, Mol. Cell. Biol. 16:4744; Barbulescu et al., 1997, Eur. J. Immunol. 27, 1098-1107; Sica et al., 1997, J. Biol. Chem. 272, 30412-30420). The production of IFN- γ is, however, normal in mice lacking ATF-2. Because cytokines are critical in the development of Th1 and Th2 cells and, thereby, in determining whether an 15 immune response will be primarily cellular or humoral, compositions and methods for modulating the production of Th1 and/or Th2 cytokines would be of tremendous benefit in modulating the immune response.

Summary of the Invention

This invention is based, at least in part, on the discovery of novel compositions 20 which act to promote the Th1 phenotype in naïve T helper precursor cells (Thp), both by initiating Th1 cell genetic programs and by repressing the opposing programs in Th2 cells. In particular, this invention provides isolated nucleic acid molecules encoding T-bet and isolated T-bet protein. T-bet (T_box expressed in T cells) is a new member of the T box family of transcription factors whose founding member is the *brachyury* gene. T-bet is constitutively expressed selectively in thymocytes and Th1 cells. T-bet is the first 25 Th1 specific transcription factor that can transactivate the interferon-gamma gene, induces interferon-gamma production in retrovirally transduced primary T cells and redirect polarized Th2 cells into the Th1 pathway. The invention also provides methods of using these novel T-bet compositions.

One aspect of the invention pertains to an isolated nucleic acid molecule comprising a nucleotide sequence encoding T-bet. In a preferred embodiment, the nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO: 1 or 3. In other embodiments, the nucleic acid molecule comprises at least 700 contiguous nucleotides of SEQ ID NO:1 or at least 500 contiguous nucleotides of SEQ ID NO:3. In a preferred embodiment, the nucleic acid molecule has at least 70 % nucleotide identity, with at least 700 contiguous nucleotides of SEQ ID NO:1 or at least 70% identity with at least 500 contiguous nucleotides of SEQ ID NO:3.

The isolated nucleic acid molecules of the invention encoding T-bet can be 10 incorporated into a vector, such as an expression vector, and this vector can be introduced into a host cell. The invention also provides a method for producing a T-bet protein by culturing a host cell of the invention (carrying a T-bet expression vector) in a suitable medium until a T-bet protein is produced. The method can further involve isolating the T-bet protein from the medium or the host cell.

15 Another aspect of the invention pertains to an isolated T-bet protein. Preferably, the T-bet protein comprises the amino acid sequence of SEQ ID NO: 2 or 4. In other embodiments, the protein has at least 60 % amino acid identity, at least 70% amino acid identity, more preferably 80 % amino identity, at, and even more preferably 90 % amino acid identity with the amino acid sequence shown in SEQ ID NO:1 or 3.

20 Fusion proteins, comprising a T-bet protein operatively linked to a polypeptide other than T-bet, are also encompassed by the invention, as well as antibodies that specifically bind a T-bet protein. The antibodies can be, for example, polyclonal antibodies or monoclonal antibodies. In one embodiment, the antibodies are coupled to a detectable substance.

25 Another aspect of the invention pertains to a nonhuman transgenic animal that contains cells carrying a transgene encoding a T-bet protein.

30 Yet another aspect of the invention pertains to a method for detecting the presence of T-bet in a biological sample. The method involves contacting the biological sample with an agent capable of detecting an indicator of T-bet activity such that the presence of T-bet is detected in the biological sample. The invention also provides a method for modulating T-bet activity in a cell comprising, involving contacting the cell

with an agent that modulates T-bet activity such that T-bet activity in the cell is modulated.

Still another aspect of the invention pertains to methods for identifying a compound that modulates the activity of a T-bet protein. These methods generally

5 involve:

providing an indicator composition that comprises a T-bet protein;
contacting the indicator composition with a test compound; and

determining the effect of the test compound on the activity of the T-bet protein in the indicator composition to thereby identify a compound that modulates the activity of

10 a T-bet protein. In a preferred embodiment, the indicator composition comprises a T-bet protein and a DNA molecule to which the T-bet protein binds and the effect of the test compound on the activity of the T-bet protein is determined by evaluating the binding of the T-bet protein to the DNA molecule in the presence and absence of the test compound. In another preferred embodiment, the indicator composition is a cell
15 comprising a T-bet protein and a reporter gene responsive to the T-bet protein and the effect of the test compound on the activity of the T-bet protein is determined by evaluating the expression of the reporter gene in the presence and absence of the test compound. In yet another embodiment, the method further involves the step of determining the effect of the test compound on an immune response to thereby identify a
20 compound that modulates an immune response.

Brief Description of the Drawings

Figure 1A shows a nucleotide sequence alignment of murine and human T-bet. The alignment was prepared using the ALIGN program. Figure 1B shows an amino acid
25 sequence alignment of murine and human T-bet prepared using the Lipman Pearson protein alignment program. The T-box sequence is shown in bold. Tyrosine phosphorylation sites are underlined. The nuclear localization site is marked with arrows.

30 Figure 2 A and B show that T-bet binds to and transactivates consensus T-box sites with functionally important domains that map to both 5' and 3' regions.

Figure 3A shows that T-bet is preferentially expressed in double negative thymocytes. Panel B shows that in a survey of Th clones, T-bet expression is restricted to Th1 cells. Panel C shows western blot analysis of T-bet. Panel D shows FACS analysis of T-bet expression..

Figure 4 A and B show that T-bet expression correlates with IFN- γ induction in NK and B cells.

10 Figure 5 shows that T-bet transactivates the IFN- γ gene in Th cells.

Figure 6 shows that retroviral gene transduction of T-bet reduces increases IFN-gamma production and represses IL-2 production.

15 Figure 7 shows that T-bet activates IFN- γ and represses IL-2 production in primary T cells.

Figure 8 shows that T-bet induces IFN-gamma and inhibits IL-4 production in developing Th2 cells.

20 Figure 9 shows that T-bet redirects polarized Th2 cells into the Th1 pathway. Th skewing was carried out as above and retroviral infections were performed on day 9 of culture

25 Figure 10 shows that T-bet redirects polarized Tc2 cells into the Tc1 pathway. CD8+ T cells were purified by MoFlo and cultured under Th2 skewing conditions as above and retroviral transductions performed on day 8 of culture.

Figure 11 shows that T-bet is tyrosine phosphorylated.

30 Figure 12 shows the activity of a T-bet dominant negative mutant.

Figure 13 shows that mutations of the T-box element of the IL-2 promoter reduce IL-2 promoter activity.

5 **Detailed Description of the Invention**

This invention pertains to T-bet compositions, such as isolated nucleic acid molecules encoding T-bet and isolated T-bet proteins, as well as methods of use therefore.

So that the invention may be more readily understood, certain terms are first 10 defined.

As used herein, the term "T-bet molecules" includes T-bet nucleic acid molecules that share structural features with the nucleic acid molecules shown in SEQ ID Nos: 1 and 3 and T-bet proteins that share the distinguishing structural and functional features of the T-bet proteins shown in SEQ ID Nos 2 and 4. The T-bet proteins are 15 members of the T-box family of proteins and share some amino acid sequence homology to Brachury, Tbx1-6, T-brain-1 (Tbr-1). T-box proteins comprise a T box domain which binds to DNA at a T box binding site. Further structural and functional features of T-bet proteins are provided below.

As used herein, the term "nucleic acid molecule" is intended to include DNA 20 molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA). The nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

As used herein, an "isolated nucleic acid molecule" refers to a nucleic acid molecule that is free of gene sequences which naturally flank the nucleic acid in the 25 genomic DNA of the organism from which the nucleic acid is derived (*i.e.*, genetic sequences that are located adjacent to the gene for the isolated nucleic molecule in the genomic DNA of the organism from which the nucleic acid is derived). For example, in various embodiments, an isolated T-bet nucleic acid molecule typically contains less than about 10 kb of nucleotide sequences which naturally flank the nucleic acid 30 molecule in genomic DNA of the cell from which the nucleic acid is derived, and more preferably contains less than about 5, kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of

naturally flanking nucleotide sequences. An "isolated" T-bet nucleic acid molecule may, however, be linked to other nucleotide sequences that do not normally flank the T-bet sequences in genomic DNA (e.g., the T-bet nucleotide sequences may be linked to vector sequences). In certain preferred embodiments, an "isolated" nucleic acid 5 molecule, such as a cDNA molecule, also may be free of other cellular material. However, it is not necessary for the T-bet nucleic acid molecule to be free of other cellular material to be considered "isolated" (e.g., a T-bet DNA molecule separated from other mammalian DNA and inserted into a bacterial cell would still be considered to be "isolated").

10 As used herein, the term "hybridizes under high stringency conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences having substantial homology (e.g., typically greater than 70% homology) to each other remain stably hybridized to each other. A preferred, non-limiting example of high stringency conditions are hybridization in a hybridization buffer that contains 6X 15 sodium chloride/ sodium citrate (SSC) at a temperature of about 45°C for several hours to overnight, followed by one or more washes in a washing buffer containing 0.2 X SSC, 0.1% SDS at a temperature of about 50-65°C.

The term "percent (%) identity" as used in the context of nucleotide and amino acid sequences (e.g., when one amino acid sequence is said to be X% identical to 20 another amino acid sequence) refers to the percentage of identical residues shared between the two sequences, when optimally aligned. To determine the percent identity of two nucleotide or amino acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps may be introduced in one sequence for optimal alignment with the other sequence). The residues at corresponding positions are then 25 compared and when a position in one sequence is occupied by the same residue as the corresponding position in the other sequence, then the molecules are identical at that position. The percent identity between two sequences, therefore, is a function of the number of identical positions shared by two sequences (*i.e.*, % identity = # of identical positions/total # of positions x 100).

Computer algorithms known in the art can be used to optimally align and compare two nucleotide or amino acid sequences to define the percent identity between the two sequences. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul 5 (1990) Proc. Natl. Acad. Sci. USA 87:2264-68, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-77. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. To obtain gapped alignments for comparison purposes. Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Research 25(17):3389-10 3402. When utilizing BLAST and Gapped BLAST programs. the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See 10 <http://www.ncbi.nlm.nih.gov>. For example, the nucleotide sequences of the invention were blasted using the default Blastn matrix 1-3 with gap penalties set at: existance 5 and extension 2. The amino acid sequences of the invention were blasted using the 15 default settings: the Blosum62 matrix with gap penalties set at existance 11 and extension 1.

Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part 20 of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. If multiple programs are used to compare sequences. the program that provides optimal alignment (i.e., the highest percent identity between the two sequences) is used for comparison purposes.

25 As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (e.g., encodes a natural protein).

As used herein, an "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, e.g., 30 complementary to the coding strand of a double-stranded cDNA molecule,

complementary to an mRNA sequence or complementary to the coding strand of a gene. Accordingly, an antisense nucleic acid can hydrogen bond to a sense nucleic acid.

As used herein, the term "coding region" refers to regions of a nucleotide sequence comprising codons which are translated into amino acid residues, whereas the 5 term "noncoding region" refers to regions of a nucleotide sequence that are not translated into amino acids (e.g., 5' and 3' untranslated regions).

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional 10 DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the 15 genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" or simply "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of 20 plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

25 As used herein, the term "host cell" is intended to refer to a cell into which a nucleic acid of the invention, such as a recombinant expression vector of the invention, has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It should be understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because 30 certain modifications may occur in succeeding generations due to either mutation or

environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

As used herein, a "transgenic animal" refers to a non-human animal, preferably a mammal, more preferably a mouse, in which one or more of the cells of the animal 5 includes a "transgene". The term "transgene" refers to exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, for example directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal.

As used herein, a "homologous recombinant animal" refers to a type of 10 transgenic non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, e.g., an embryonic cell of the animal, prior to development of the animal.

As used herein, an "isolated protein" refers to a protein that is substantially free 15 of other proteins, cellular material and culture medium when isolated from cells or produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized.

As used herein, the term "antibody" is intended to include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., 20 molecules that contain an antigen binding site which specifically binds (immunoreacts with) an antigen, such as Fab and F(ab')₂ fragments. The terms "monoclonal antibodies" and "monoclonal antibody composition", as used herein, refer to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of an antigen, whereas the term "polyclonal 25 antibodies" and "polyclonal antibody composition" refer to a population of antibody molecules that contain multiple species of antigen binding sites capable of interacting with a particular antigen. A monoclonal antibody compositions thus typically display a single binding affinity for a particular antigen with which it immunoreacts.

There is a known and definite correspondence between the amino acid sequence 30 of a particular protein and the nucleotide sequences that can code for the protein, as defined by the genetic code (shown below). Likewise, there is a known and definite

correspondence between the nucleotide sequence of a particular nucleic acid molecule and the amino acid sequence encoded by that nucleic acid molecule, as defined by the genetic code.

5 GENETIC CODE

Alanine (Ala, A)	GCA, GCC, GCG, GCT
Arginine (Arg, R)	AGA, ACG, CGA, CGC, CGG, CGT
Asparagine (Asn, N)	AAC, AAT
Aspartic acid (Asp, D)	GAC, GAT
10 Cysteine (Cys, C)	TGC, TGT
Glutamic acid (Glu, E)	GAA, GAG
Glutamine (Gln, Q)	CAA, CAG
Glycine (Gly, G)	GGA, GGC, GGG, GGT
Histidine (His, H)	CAC, CAT
15 Isoleucine (Ile, I)	ATA, ATC, ATT
Leucine (Leu, L)	CTA, CTC, CTG, CTT, TTA, TTG
Lysine (Lys, K)	AAA, AAG
Methionine (Met, M)	ATG
Phenylalanine (Phe, F)	TTC, TTT
20 Proline (Pro, P)	CCA, CCC, CCG, CCT
Serine (Ser, S)	AGC, AGT, TCA, TCC, TCG, TCT
Threonine (Thr, T)	ACA, ACC, ACG, ACT
Tryptophan (Trp, W)	TGG
Tyrosine (Tyr, Y)	TAC, TAT
25 Valine (Val, V)	GTA, GTC, GTG, GTT
Termination signal (end)	TAA, TAG, TGA

An important and well known feature of the genetic code is its redundancy, whereby, for most of the amino acids used to make proteins, more than one coding nucleotide triplet 30 may be employed (illustrated above). Therefore, a number of different nucleotide sequences may code for a given amino acid sequence. Such nucleotide sequences are considered functionally equivalent since they result in the production of the same amino acid sequence in all organisms (although certain organisms may translate some sequences more efficiently than they do others). Moreover, occasionally, a methylated 35 variant of a purine or pyrimidine may be found in a given nucleotide sequence. Such

methylations do not affect the coding relationship between the trinucleotide codon and the corresponding amino acid.

In view of the foregoing, the nucleotide sequence of a DNA or RNA molecule coding for a T-bet protein of the invention (or any portion thereof) can be used to derive

5 the T-bet amino acid sequence, using the genetic code to translate the DNA or RNA molecule into an amino acid sequence. Likewise, for any T-bet-amino acid sequence, corresponding nucleotide sequences that can encode the T-bet protein can be deduced from the genetic code (which, because of its redundancy, will produce multiple nucleic acid sequences for any given amino acid sequence). Thus, description and/or disclosure

10 herein of a T-bet nucleotide sequence should be considered to also include description and/or disclosure of the amino acid sequence encoded by the nucleotide sequence. Similarly, description and/or disclosure of a T-bet amino acid sequence herein should be considered to also include description and/or disclosure of all possible nucleotide sequences that can encode the amino acid sequence.

15 *Brachyury* or *T* is the founding member of a family of transcription factors that share a 200 amino acid DNA-binding domain called the T-box (reviewed in (Smith, 1997; Papaioannou, 1997; Meisler, 1997)). The *Brachyury* (Greek for 'short tail') mutation was first described in 1927 in heterozygous mutant animals who had a short, slightly kinked tail (Herrmann et al., 1990). The amino-terminal half (amino acids 1-20 229) of the Brachyury T-box protein contains a conserved domain known as the T box which has been shown to exhibit sequence-specific DNA-binding activity (Kispert, A. & Herrmann, B. G. 1993. *EMBO J.* 12:3211; Papapetrou, C., et al. 1997. *FEBS Lett.* 409:201; Kispert, A., et al. 1995. *EMBO J.* 14:4763). The C-terminal half contains two pairs of transactivation and repression domains. The similarity of sequence between the

25 T box region in orthologous species can be as high as 99% and is around 40-70% between non-orthologous genes. The T-box domain has recently been co-crystallized with DNA and demonstrates a novel sequence-specific DNA recognition architecture in which the protein contacts DNA in both the major and minor grooves (Müller, C. W. & Herrmann, B. G. 1997. *Nature* 389, 884.

A yeast one hybrid approach was used to identify Th-1 specific transcription factors. Yeast cells were made to express an IL-2 promoter-reporter gene construct and were transformed with a cDNA library made from an anti-CD3 activated Th1 cell clone. Inspection of the IL-2 promoter reveals an excellent T-box binding site at -240 to -220 5 just 5' of the NF_κB site. As described in the appended examples, T-bet was isolated in a yeast one hybrid screening assay based on its ability to bind to the IL-2 promoter.

The nucleotide sequence encoding murine T-bet is shown in SEQ ID NO:3. Murine T-bet is a 530 amino acid protein with a 190 amino acid T-box domain located at residues 136-326. The amino acid sequence of murine T-bet is shown in SEQ ID 10 NO:4. After the murine T-bet sequence was cloned as described herein, it was possible to compile the sequence of the human ortholog of T-bet from nucleic acid fragments which were not previously known to encode any known protein. The nucleotide sequence of human T-bet is shown in SEQ ID NO:1. Human T-bet is a 535 amino acid protein with a 190 amino acid T-box domain located at residues 138-327. The human T- 15 bet gene maps to chromosome 17. The nucleotide and amino acid sequences of two members (human and mouse) of the T-bet family of proteins are shown in Figure 1 and SEQ ID Nos: 1-4.

The T-bet proteins of the invention have homology to T-box proteins. There are now eight T-box genes in the mouse not including *Brachyury*. These include Tbx1-6, T- 20 brain-1 (Tbr-1) and now, T-bet, each with a distinct and usually complex expression pattern. T-brain-1 expression, for example is largely restricted to distinct domains within the cerebral cortex (Bulfone, A..et al. 1995. *Neuron* 15, 63. T-bet is most similar in sequence to Tbr-1. Outside of the T-box, the T-bet proteins of the invention bear no similarity to other T-box proteins.

25 T-bet is T-box protein expressed only in T cells and is most similar in sequence to Tbr-1. Other species also express *Brachyury*-like genes. Such vertebrate species include Xenopus, zebrafish, chick and humans (Rao, 1994; Horb and Thomsen, 1997; Conlon et al., 1996; Ryan et al., 1996; Schulte-Merker et al., 1994; Edwards et al., 1996; Morrison et al., 1996; Law et al., 1995; Cambell et al., 1998) as well as more distant 30 species such as amphioxus, ascidians, echinoderms, *Caenorhabditis elegans*, *Drosophila*

and other insects (Holland et al., 1995). These genes are conserved both in sequence and in expression pattern.

T-bet is unique in that it is the only T-box protein to be tyrosine phosphorylated. There are two consensus tyrosine phosphorylation sites at aa 328-336 and 526-534 of

5 human T-bet and 327-335 and 521-529 of murine T-bet. A nuclear localization sequence is also present at amino acids 498-501 of human T-bet and 493-496 of murine T-bet. Mapping experiments locate two transactivation domains, one 5' and one 3' of the T-box domain. The data shown herein demonstrate that T-bet binds to a consensus T-box site (defined by target site selection *in vitro* as 5'-

10 GGGATTTCACACCTAGGTGTGAAATTCCC-3') and to a T-box site in the IL-2 promoter. T-bet is expressed only in the thymus and in the peripheral lymphoid system. In the periphery, T-bet is expressed only in Th1 cells where it is induced both in response to TcR stimulation and to IL-12. In the thymus levels of T-bet are highest in DN and Rag2-/-thymocytes.

15 These data demonstrate that the selective expression of T-bet, a novel T-box family member, accounts for tissue-specific IFN- γ expression. T-bet is expressed only in Th1 and not in Th2 cells and is induced in the former upon transmission of signals through the T cell receptor. The expression of T-bet correlates with IFN- γ expression in Th1 cells, NK cells and B cells, and T-bet is a potent transactivator of the IFN- γ gene.

20 Most convincing, retroviral mediated transduction of Thp, Th1 and polarized Th2 and Tc2 cells with T-bet results in an impressive induction of IFN- γ expression. This is accompanied by repression of both IL-2 and IL-4 production. Thus, the function of T-bet extends beyond the simple control of IFN- γ gene transcription. T-bet converts both polarized effector Th2 cells and polarized Tc2 cells into the opposing Th1 and Tc1

25 subsets, respectively. Taken together, these data demonstrate that T-bet is responsible for the genetic program that initiates Th1 lineage development from naïve Thp cells and acts both by initiating Th1 genetic programs and by repressing the opposing programs in Th2 cells.

Various aspects of the invention are described in further detail in the following subsections:

1. Isolated Nucleic Acid Molecules

5 One aspect of the invention pertains to isolated nucleic acid molecules that encode T-bet. In a preferred embodiment, the nucleic acid molecule of the invention comprises the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:3. In another embodiment, a nucleic acid molecule of the invention comprises at least about 700 contiguous nucleotides of SEQ ID NO:1 or at least about 500 contiguous nucleotides of 10 SEQ ID NO:3. In a preferred embodiment, a nucleic acid molecule of the invention comprises at least about 800, at least about 1000, at least about 1200, at least about 1400 or at least about 1600 contiguous nucleotides of SEQ ID NO:1. In another preferred embodiment, a nucleic acid molecule of the invention comprises at least about 600, at least about 800, at least about 1000, at least about 1200, or at least about 1400 15 contiguous nucleotides of SEQ ID NO:3.

In other embodiments, the nucleic acid molecule has at least 70 % identity, more preferably 80% identity, and even more preferably 90% identity with a nucleic acid molecule comprising: at least about 700, at least about 800, at least about 1000, at least about 1200, at least about 1400 or at least about 1600 contiguous nucleotides of SEQ ID

20 NO:1. In other embodiments, the nucleic acid molecule has at least 70 % identity, more preferably 80% identity, and even more preferably 90% nucleotide identity with a nucleic acid molecule comprising: at least about 600, at least about 800, at least about 1000, at least about 1200, or at least about 1400 contiguous nucleotides of SEQ ID NO:3.

25 Nucleic acid molecules that differ from SEQ ID NO: 1 or 3 due to degeneracy of the genetic code, and thus encode the same T-bet protein as that encoded by SEQ ID NO: 1 and 3, are encompassed by the invention. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention has a nucleotide sequence encoding a protein having an amino acid sequence shown in SEQ ID NO: 2 or SEQ ID NO:4.

In addition, nucleic acid molecules encoding T-bet proteins can be isolated from other sources using standard molecular biology techniques and the sequence information provided herein. For example, a T-bet DNA can be isolated from a human genomic DNA library using all or portion of SEQ ID NO:1 or 3 as a hybridization probe and

5 standard hybridization techniques (e.g., as described in Sambrook, J., *et al. Molecular Cloning: A Laboratory Manual*, 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). Moreover, a nucleic acid molecule encompassing all or a portion of a T-bet gene can be isolated by the polymerase chain reaction using oligonucleotide primers designed based upon the sequence of SEQ ID NO: 1 or 3. For example, mRNA

10 can be isolated from cells (e.g., by the guanidinium-thiocyanate extraction procedure of Chirgwin *et al.* (1979) *Biochemistry* 18: 5294-5299) and cDNA can be prepared using reverse transcriptase (e.g., Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD; or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for PCR

15 amplification can be designed based upon the nucleotide sequence shown in SEQ ID NO: 1 or 3. A nucleic acid of the invention can be amplified using cDNA or, alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis.

20 Furthermore, oligonucleotides corresponding to a T-bet nucleotide sequence can be prepared by standard synthetic techniques, e.g., using an automated DNA synthesizer.

In addition to the T-bet nucleotide sequence shown in SEQ ID NO: 1 and 3, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to minor changes in the nucleotide or amino acid sequences of T-bet may exist

25 within a population. Such genetic polymorphism in the T-bet gene may exist among individuals within a population due to natural allelic variation. Such natural allelic variations can typically result in 1-2 % variance in the nucleotide sequence of the a gene. Any and all such nucleotide variations and resulting amino acid polymorphisms in T-bet that are the result of natural allelic variation and that do not alter the functional activity

30 of T-bet are intended to be within the scope of the invention.

Nucleic acid molecules corresponding to natural allelic variants of the T-bet DNAs of the invention can be isolated based on their homology to the T-bet nucleic acid molecules disclosed herein using the human DNA, or a portion thereof, as a hybridization probe according to standard hybridization techniques under high

- 5 stringency hybridization conditions. Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention hybridizes under high stringency conditions to a second nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 1 or 3. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under high stringency conditions to the sequence of SEQ ID NO: of SEQ ID NO: 1 or 3. In one
- 10 embodiment, such a nucleic acid molecule is at least about 700, 800, 900, 1000, 1200, 1300, 1400, 1500, or 1600 nucleotides in length. In another embodiment, such a nucleic acid molecule and comprises at least about 700, 800, 900, 1000, 1200, 1300, 1400, 1500, or 1600 contiguous nucleotides of SEQ ID NO: 1 or at least about 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, or 1500 contiguous nucleotides of SEQ ID NO: 3.
- 15 Preferably, an isolated nucleic acid molecule corresponds to a naturally-occurring allelic variant of a T-bet nucleic acid molecule.

In addition to naturally-occurring allelic variants of the T-bet sequence that may exist in the population, the skilled artisan will further appreciate that minor changes may be introduced by mutation into the nucleotide sequence of SEQ ID NO: 1 or 3, thereby leading to changes in the amino acid sequence of the encoded protein, without altering the functional activity of the T-bet protein. For example, nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues may be made in the sequence of SEQ ID NO: 1 or 3. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of T-bet (e.g., the sequence of SEQ ID NO: 1 or 3) without altering the functional activity of T-bet, such as its ability to interact with DNA or its ability to enhance transcription from an IFN-gamma promoter, whereas an "essential" amino acid residue is required for functional activity.

Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding T-bet proteins that contain changes in amino acid residues that are not essential for T-bet activity. Such T-bet proteins differ in amino acid sequence from SEQ ID NO: 2 or 4 yet retain T-bet activity. An isolated nucleic acid molecule encoding a non-

natural variant of a T-bet protein can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of SEQ ID NO: 1 or 3 such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced into SEQ ID NO: 1 or 3 by standard 5 techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, 10 including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., 15 tyrosine, phenylalanine, tryptophan, histidine). Thus, a nonessential amino acid residue in T-bet is preferably replaced with another amino acid residue from the same side chain family.

Alternatively, in another embodiment, mutations can be introduced randomly along all or part of the T-bet coding sequence, such as by saturation mutagenesis, and 20 the resultant mutants can be screened for their ability to bind to DNA and/or activate transcription, to identify mutants that retain functional activity. Following mutagenesis, the encoded T-bet mutant protein can be expressed recombinantly in a host cell and the functional activity of the mutant protein can be determined using assays available in the art for assessing T-bet activity (e.g., by measuring the ability of the protein to bind to a 25 T-box binding element present in DNA or by measuring the ability of the protein to modulate a Th1 or Th2 phenotype in a T cell).

Another aspect of the invention pertains to isolated nucleic acid molecules that are antisense to the coding strand of a T-bet mRNA or gene. An antisense nucleic acid 30 of the invention can be complementary to an entire T-bet coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a coding region of the coding strand of a nucleotide sequence encoding T-bet that is

unique to the T-bet family of proteins or which is unique to a T-bet sequence from a particular species. In another embodiment, the antisense nucleic acid molecule is antisense to a noncoding region of the coding strand of a nucleotide sequence encoding T-bet that is unique to T-bet family of proteins or which is unique to a T-bet sequence

5 from a particular species. In preferred embodiments, an antisense molecule of the invention comprises at least about 700 contiguous nucleotides of the noncoding strand of SEQ ID NO: 1, more preferably at least 800, 1000, 1200, 1400, or 1600 contiguous nucleotides of the noncoding strand of SEQ ID NO: 1 or at least about 500 contiguous nucleotides of the noncoding strand of SEQ ID NO: 3, more preferably at least 600,

10 800, 1000, 1200, or 1400 contiguous nucleotides of the noncoding strand of SEQ ID NO: 3.

Given the coding strand sequences encoding T-bet disclosed herein (e.g., SEQ ID NOs: 1 and 3, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule may be

15 complementary to the entire coding region of T-bet mRNA, or alternatively can be an oligonucleotide which is antisense to only a portion of the coding or noncoding region of T-bet mRNA. For example, the antisense oligonucleotide may be complementary to the region surrounding the translation start site of T-bet mRNA. An antisense oligonucleotide can be, for example, about 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in

20 length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical

25 stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense

30 orientation to a target nucleic acid of interest, described further in the following subsection).

In another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. A ribozyme having specificity for a T-bet-encoding nucleic acid

5 can be designed based upon the nucleotide sequence of a T-bet gene disclosed herein. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the base sequence of the active site is complementary to the base sequence to be cleaved in a T-bet-encoding mRNA. See for example Cech *et al.* U.S. Patent No. 4,987,071; and Cech *et al.* U.S. Patent No. 5,116,742. Alternatively, T-bet mRNA can be used to select

10 a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See for example Bartel, D. and Szostak, J.W. (1993) *Science* 261: 1411-1418.

Yet another aspect of the invention pertains to isolated nucleic acid molecules encoding T-bet fusion proteins. Such nucleic acid molecules, comprising at least a first nucleotide sequence encoding a T-bet protein, polypeptide or peptide operatively linked

15 to a second nucleotide sequence encoding a non-T-bet protein, polypeptide or peptide, can be prepared by standard recombinant DNA techniques. T-bet fusion proteins are described in further detail below in subsection III.

II. Recombinant Expression Vectors and Host Cells

20 Another aspect of the invention pertains to vectors, preferably recombinant expression vectors, containing a nucleic acid encoding T-bet (or a portion thereof). The expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on

25 the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an *in vitro* transcription/translation system or in a host cell when the

30 vector is introduced into the host cell). The term "regulatory sequence" includes promoters, enhancers and other expression control elements (e.g., polyadenylation

signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the 5 nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion 10 proteins or peptides, encoded by nucleic acids as described herein (e.g., T-bet proteins, mutant forms of T-bet proteins, T-bet fusion proteins and the like).

The recombinant expression vectors of the invention can be designed for expression of T-bet protein in prokaryotic or eukaryotic cells. For example, T-bet can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression 15 vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector may be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

20 Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors can serve one or more purposes: 1) to increase expression of recombinant 25 protein; 2) to increase the solubility of the recombinant protein; 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification; 4) to provide an epitope tag to aid in detection and/or purification of the protein; and/or 5) to provide a marker to aid in detection of the protein (e.g., a color marker using β -galactosidase fusions). Often, in fusion expression vectors, a proteolytic cleavage site is 30 introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification

of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc.; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which 5 fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein. Recombinant proteins also can be expressed in eukaryotic cells as fusion proteins for the same purposes discussed above.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral 10 polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the 20 recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, (1992) *Nuc. Acids Res.* 20:2111-2118). 25 Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the T-bet expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYEpSec1 (Baldari, *et al.*, (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-30 943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA).

Alternatively, T-bet can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith *et al.* (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pMex-Neol, pCDM8 (Seed, B., (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987), *EMBO J.* 6:187-195). When used in mammalian cells, 10 the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type 15 (e.g., tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) 20 *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *Proc. Natl. Acad. Sci. USA* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (e.g., milk whey promoter; U.S. Patent No. 25 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α -fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

Moreover, inducible regulatory systems for use in mammalian cells are known in 30 the art, for example systems in which gene expression is regulated by heavy metal ions (see e.g., Mayo *et al.* (1982) *Cell* 29:99-108; Brinster *et al.* (1982) *Nature* 296:39-42;

Searle *et al.* (1985) *Mol. Cell. Biol.* 5:1480-1489). heat shock (see e.g., Nouer *et al.* (1991) in *Heat Shock Response*, e.d. Nouer, L., CRC, Boca Raton, FL, pp167-220), hormones (see e.g., Lee *et al.* (1981) *Nature* 294:228-232; Hynes *et al.* (1981) *Proc. Natl. Acad. Sci. USA* 78:2038-2042; Klock *et al.* (1987) *Nature* 329:734-736; Israel &

5 Kaufman (1989) *Nucl. Acids Res.* 17:2589-2604; and PCT Publication No. WO 93/23431), FK506-related molecules (see e.g., PCT Publication No. WO 94/18317) or tetracyclines (Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Gossen, M. *et al.* (1995) *Science* 268:1766-1769; PCT Publication No. WO 94/29442; and PCT Publication No. WO 96/01313). Accordingly, in another

10 embodiment, the invention provides a recombinant expression vector in which T-bet DNA is operatively linked to an inducible eukaryotic promoter, thereby allowing for inducible expression of T-bet protein in eukaryotic cells.

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to T-bet mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for 20 instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell 25 type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. *et al.*, Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to recombinant host cells into which a vector, preferably a recombinant expression vector, of the invention has been introduced. 30 A host cell may be any prokaryotic or eukaryotic cell. For example, T-bet protein may be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells

(such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art. Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of 5 art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook *et al.* (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and 10 other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is 15 generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker may be introduced into a host cell on the same vector as that encoding T-bet or may be introduced on a separate vector. Cells stably transfected with the introduced nucleic 20 acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) T-bet protein. Accordingly, the invention further provides methods for producing T-bet protein using the host cells of the 25 invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding T-bet has been introduced) in a suitable medium until T-bet is produced. In another embodiment, the method further comprises isolating T-bet from the medium or the host cell. In its native form the T-bet protein is an intracellular protein and, accordingly, recombinant T-bet 30 protein can be expressed intracellularly in a recombinant host cell and then isolated from the host cell, *e.g.* by lysing the host cell and recovering the recombinant T-bet protein

from the lysate. Alternatively, recombinant T-bet protein can be prepared as a extracellular protein by operatively linking a heterologous signal sequence to the amino-terminus of the protein such that the protein is secreted from the host cells. In this case, recombinant T-bet protein can be recovered from the culture medium in which the cells 5 are cultured.

Certain host cells of the invention can also be used to produce nonhuman transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which T-bet-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic 10 animals in which exogenous T-bet sequences have been introduced into their genome or homologous recombinant animals in which endogenous T-bet sequences have been altered. Such animals are useful for studying the function and/or activity of T-bet and for identifying and/or evaluating modulators of T-bet activity. Accordingly, another aspect of the invention pertains to nonhuman transgenic animals which contain cells 15 carrying a transgene encoding a T-bet protein or a portion of a T-bet protein. In a subembodiment, of the transgenic animals of the invention, the transgene alters an endogenous gene encoding an endogenous T-bet protein (e.g., homologous recombinant animals in which the endogenous T-bet gene has been functionally disrupted or "knocked out", or the nucleotide sequence of the endogenous T-bet gene has been 20 mutated or the transcriptional regulatory region of the endogenous T-bet gene has been altered).

A transgenic animal of the invention can be created by introducing T-bet-encoding nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, and allowing the oocyte to develop in a pseudopregnant female foster 25 animal. The T-bet nucleotide sequence of SEQ ID NO: 1 or 3 can be introduced as a transgene into the genome of a non-human animal. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to the T-bet transgene to direct expression of T-bet protein to particular cells. 30 Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are

described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder *et al.*, U.S. Patent No. 4,873,191 by Wagner *et al.* and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A 5 transgenic founder animal can be identified based upon the presence of the T-bet transgene in its genome and/or expression of T-bet mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding T-bet can further be bred to other transgenic animals carrying other transgenes.

10 To create a homologous recombinant animal, a vector is prepared which contains at least a portion of a T-bet gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the endogenous T-bet gene. In one embodiment, a homologous recombination vector is designed such that, upon homologous recombination, the endogenous T-bet gene is functionally disrupted (*i.e.*, no 15 longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous T-bet gene replaced by the T-bet gene. In the homologous recombination vector, the altered portion of the T-bet gene is flanked at its 5' and 3' ends by additional nucleic acid of the T-bet gene to allow for homologous recombination to 20 occur between the exogenous T-bet gene carried by the vector and an endogenous T-bet gene in an embryonic stem cell. The additional flanking T-bet nucleic acid is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see *e.g.*, Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503 for a 25 description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced T-bet gene has homologously recombined with the endogenous T-bet gene are selected (see *e.g.*, Li, E. *et al.* (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (see *e.g.*, Bradley, 30 A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be

implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing 5 homologous recombination vectors and homologous recombinant animals are described further in Bradley, A. (1991) *Current Opinion in Biotechnology* 2:823-829 and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec *et al.*; WO 91/01140 by Smithies *et al.*; WO 92/0968 by Zijlstra *et al.*; and WO 93/04169 by Berns *et al.*

In addition to the foregoing, the skilled artisan will appreciate that other 10 approaches known in the art for homologous recombination can be applied to the instant invention. Enzyme-assisted site-specific integration systems are known in the art and can be applied to integrate a DNA molecule at a predetermined location in a second target DNA molecule. Examples of such enzyme-assisted integration systems include the Cre recombinase-lox target system (e.g., as described in Baubonis, W. and Sauer, B. 15 (1993) *Nucl. Acids Res.* 21:2025-2029; and Fukushige, S. and Sauer, B. (1992) *Proc. Natl. Acad. Sci. USA* 89:7905-7909) and the FLP recombinase-FRT target system (e.g., as described in Dang, D.T. and Perrimon, N. (1992) *Dev. Genet.* 13:367-375; and Fiering, S. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:8469-8473). Tetracycline-regulated inducible homologous recombination systems, such as described in PCT 20 Publication No. WO 94/29442 and PCT Publication No. WO 96/01313, also can be used.

In another embodiment, transgenic animals can be made in which T-bet is 25 expressed in all T cells, e.g., using the CD4 enhancer (Zheng, W.-P. & Flavell, R. A. 1997. *Cell* 89, 587). Recent work suggests the CD2 enhancer can also be used. In fact, it is more powerful in achieving high level expression in T cells. expression is not variegated and transgene expression is copy number-dependent (Zhumabekov, T., *et al.* 1995. *J. Immunol. Meth.* 185, 133; Sharp, L. L.. *et al.* 1997. *Immunity* 7, 609). Mice with high level expression of T-bet RNA (using the human growth hormone intron as a probe to distinguish transgene driven T-bet RNA from endogenous T-bet) can be identified by 30 screening adequate numbers of founders.

In another approach, a dominant repressor transgenic can be created. For example, a dominant-repressor T-bet can be made by using the proximal lck enhancer (Alberola-Ila, J., et al. 1996 *J. Exp. Med.* 184, 9) driving a fusion of T-bet and engrailed can be made (Taylor, D., 1996. *Genes Dev.* 10, 2732; Li, J., Thurm, H., et al. 1997.

5 *Proc. Natl. Acad. Sci. USA* 94, 10885). This construct specifically represses T-bet transactivation of a multimerized T-bet reporter and does not affect NFAT-dependent reporter transactivation.

Alternatively, null mutations can be generated by targeted mutagenesis in ES cells (Ranger, A. M., et al. 1998. *Nature* 392, 186; Hodge, M. R., et al. 1996. *Immunity*

10 4:1., 144; Grusby, M. J., et al. 1991. *Science* 253, 1417; Reimold, A. M., et al. 1996. *Nature* 379: 262; Kaplan, M. H., 1996. *Immunity* :313; Kaplan, M. H., et al. 1996. *Nature* 382, 174; Smiley, S. T., et al. 1997. *Science* 275, 977). For example using techniques which are known in the art, a genomic T-bet clone can be isolated from a genomic library, the intron-exon organization delineated, and a targeting construct in the
15 cre-lox vector (see discussion below) created which should delete the first exon and 450 bp of upstream promoter sequence. This construct can be electroporated into an ES cell line, and double drug resistant (e.g., neomycin, gancyclovir) clones identified by Southern blot analysis. Clones bearing homologous recombinant events in the T-bet locus can then be identified and injected into blastocysts obtained from day 3.5 BALB/c
20 pregnant mice. Chimeric mice can then be produced and mated to wildtype BALB/c mice to generate germline transmission of the disrupted T-bet gene.

In another embodiment, implantation into RAG2-deficient blastocysts (Chen, J., et al. 1993. *Proc. Natl. Acad. Sci. USA* 90, 4528) or the cre-lox inducible deletion approach can be used to develop mice that are lacking T-bet only in the immune system.

25 For example, the targeting construct can be made in the cre-lox vector. The blastocyst complementation system has been used to study NFATc, an embryonic lethal phenotype (Ranger, A. M., et al. 1998. *Immunity* 8:125). This approach requires disrupting the T-bet gene on both chromosomes in ES cells, which can be accomplished, e.g., by using a mutant neomycin gene and raising the concentration of G418 in the ES cultures, as
30 described (Chen, J., 1993. *Proc. Natl. Acad. Sci. USA* 90:4528) or by flanking the neo gene with cre-lox sites. To disrupt the second allele, the neomycin gene can be deleted

by transfecting the ES clone with the cre recombinase, and then the ES clone can be retransfected with the same targeting construct to select clones with T-bet deletions on both alleles. A third transfection with cre-recombinase yields the desired doubly-deficient ES cells. Such doubly targeted ES cells are then implanted into RAG2

5 blastocysts and the lymphoid organs of the chimeric mice thus generated will be entirely colonized by the transferred ES cells. This allows assessment of the effect of the absence of T-bet on cells of the lymphoid system without affecting other organ systems where the absence of T-bet might cause lethality.

The conditional ablation approach employing the cre-lox system can also be used. Briefly, a targeting construct is generated in which lox recombination sequences are placed in intronic regions flanking the exons to be deleted. This construct is then transfected into ES cells and mutant mice are generated as above. The resulting mutant mice are then mated to mice transgenic for the cre recombinase driven by an inducible promoter. When cre is expressed, it induces recombination between the introduced lox 15 sites in the T-bet gene, thus effectively disrupting gene function. The key feature of this approach is that gene disruption can be induced in the adult animal at will by activating the cre recombinase.

A tissue-specific promoter can be used to avoid abnormalities in organs outside the immune system. The cre-expressing transgene may be driven by an inducible 20 promoter. Several inducible systems are now being used in cre-lox recombination strategies, the most common being the tetracycline and ecdysone systems. A tissue-specific inducible promoter can be used if there is embryonic lethality in the T-bet null mouse.

An alternative approach is to generate a transgenic mouse harboring a regulated 25 T-bet gene (for example using the tetracycline off promoter; e.g., St-Onge, et al. 1996. *Nuc. Acid Res.* 24: 3875-3877) and then breed this transgenic to the T-bet deficient mouse. This approach permits creation of mice with normal T-bet function; tetracycline can be administered to adult animals to induce disruption of T-bet function in peripheral T cells, and then the effect of T-bet deficiency can be examined over time. Repeated 30 cycles of provision and then removal of drug (tetracycline) permits turning the T-bet gene on and off at will.

III. Isolated T-bet Proteins and Anti-T-bet Antibodies

Another aspect of the invention pertains to isolated T-bet proteins. Preferably, the T-bet protein comprises the amino acid sequence encoded by SEQ ID NO:1 or 3. In another preferred embodiment, the protein comprises the amino acid sequence of SEQ 5 ID NO: 2 or 4. In other embodiments, the protein has at least 60 % amino acid identity, more preferably 70% amino acid identity, more preferably 80%, and even more preferably, 90% or 95% amino acid identity with the amino acid sequence shown in SEQ ID NO: 2 or 4.

In other embodiments, the invention provides isolated portions of the T-bet 10 protein. For example, the invention further encompasses an amino-terminal portion of T-bet that includes a T-box domain. In various embodiments, this amino terminal portion encompasses at least amino acids 138-327 of human T-bet or at least amino acids 137-326 of mouse T-bet. Another isolated portion of T-bet provided by the invention is a portion encompassing a tyrosine phosphorylation site. This portion 15 encompasses at least amino acids 324-366 and/or 523-534 of human T-bet or amino acids 323-335 or 518-529 of murine T-bet. Yet another isolated portion of T-bet provided herein is a portion encompassing a nuclear localization sequence shown in amino acids 498-501 of human T-bet or 493-496 of murine T-bet.

T-bet proteins of the invention are preferably produced by recombinant DNA 20 techniques. For example, a nucleic acid molecule encoding the protein is cloned into an expression vector (as described above), the expression vector is introduced into a host cell (as described above) and the T-bet protein is expressed in the host cell. The T-bet protein can then be isolated from the cells by an appropriate purification scheme using standard protein purification techniques. Alternative to recombinant expression, a T-bet 25 polypeptide can be synthesized chemically using standard peptide synthesis techniques. Moreover, native T-bet protein can be isolated from cells (e.g., from T cells), for example by immunoprecipitation using an anti-T-bet antibody.

The invention also provides T-bet fusion proteins. As used herein, a T-bet "fusion protein" comprises a T-bet polypeptide operatively linked to a polypeptide other 30 than T-bet. A "T-bet polypeptide" refers to a polypeptide having an amino acid sequence corresponding to T-bet protein, or a peptide fragment thereof which is unique

to T-bet protein whereas a "polypeptide other than T-bet" refers to a polypeptide having an amino acid sequence corresponding to another protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the T-bet polypeptide and the other polypeptide are fused in-frame to each other. The other polypeptide may be fused to the

5 N-terminus or C-terminus of the T-bet polypeptide. For example, in one embodiment, the fusion protein is a GST-T-bet fusion protein in which the T-bet sequences are fused to the C-terminus of the GST sequences. In another embodiment, the fusion protein is a T-bet-HA fusion protein in which the T-bet nucleotide sequence is inserted in a vector such as pCEP4-HA vector (Herrschner, R.F. *et al.* (1995) *Genes Dev.* 9:3067-3082) such

10 that the T-bet sequences are fused in frame to an influenza hemagglutinin epitope tag. Such fusion proteins can facilitate the purification of recombinant T-bet.

Preferably, a T-bet fusion protein of the invention is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR

15 amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that

20 already encode a fusion moiety (e.g., a GST polypeptide or an HA epitope tag). A T-bet-encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the T-bet protein.

An isolated T-bet protein, or fragment thereof, can be used as an immunogen to generate antibodies that bind specifically to T-bet using standard techniques for

25 polyclonal and monoclonal antibody preparation. The T-bet protein can be used to generate antibodies. For example, polyclonal antisera, can be produced in rabbits using

full-length recombinant bacterially produced T-bet as the immunogen. This same immunogen can be used to produce mAb by immunizing mice and removing spleen cells from the immunized mice. Spleen cells from mice mounting an immune response to T-bet can be fused to myeloma cells, e.g., SP2/O-Ag14 myeloma. As described in the 5 appended examples, this methods were used to make polyclonal and monoclonal antibodies which bind to T-bet.

Alternatively, an antigenic peptide fragment of T-bet can be used as the immunogen. An antigenic peptide fragment of T-bet typically comprises at least 8 amino acid residues of the amino acid sequence shown in SEQ ID NO: 2 or 4 and 10 encompasses an epitope of T-bet such that an antibody raised against the peptide forms a specific immune complex with T-bet. Preferably, the antigenic peptide comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues. Preferred epitopes encompassed by the antigenic peptide are regions of T-bet 15 that are located on the surface of the protein, e.g., hydrophilic regions, and that are unique to T-bet. In one embodiment such epitopes can be specific for T-bet proteins from one species, such as mouse or human (i.e., an antigenic peptide that spans a region of T-bet that is not conserved across species is used as immunogen; such non conserved residues can be determined using an alignment such as that provided herein). A standard 20 hydrophobicity analysis of the T-bet protein can be performed to identify hydrophilic regions.

A T-bet immunogen typically is used to prepare antibodies by immunizing a suitable subject, (e.g., rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for examples, recombinantly 25 expressed T-bet protein or a chemically synthesized T-bet peptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory agent. Immunization of a suitable subject with an immunogenic T-bet preparation induces a polyclonal anti-T-bet antibody response.

Accordingly, another aspect of the invention pertains to anti-T-bet antibodies. 30 Polyclonal anti-T-bet antibodies can be prepared as described above by immunizing a suitable subject with a T-bet immunogen. The anti-T-bet antibody titer in the

immunized subject can be monitored over time by standard techniques, such as with an enzyme linked immunosorbent assay (ELISA) using immobilized T-bet. If desired, the antibody molecules directed against T-bet can be isolated from the mammal (e.g., from the blood) and further purified by well known techniques, such as protein A

5 chromatography to obtain the IgG fraction. At an appropriate time after immunization, e.g., when the anti-T-bet antibody titers are highest, antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler and Milstein (1975, *Nature* 256:495-497) (see also, Brown *et al.* (1981) *J. Immunol* 127:539-
10 46; Brown *et al.* (1980) *J Biol Chem* 255:4980-83; Yeh *et al.* (1976) *PNAS* 76:2927-31; and Yeh *et al.* (1982) *Int. J. Cancer* 29:269-75), the more recent human B cell hybridoma technique (Kozbor *et al.* (1983) *Immunol Today* 4:72), the EBV-hybridoma technique (Cole *et al.* (1985). *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) or trioma techniques. The technology for producing monoclonal
15 antibody hybridomas is well known (see generally R. H. Kenneth, in *Monoclonal Antibodies: A New Dimension In Biological Analyses*, Plenum Publishing Corp., New York, New York (1980); E. A. Lerner (1981) *Yale J. Biol. Med.*, 54:387-402; M. L. Gefter *et al.* (1977) *Somatic Cell Genet.*, 3:231-36). Briefly, an immortal cell line (typically a myeloma) is fused to lymphocytes (typically splenocytes) from a mammal
20 immunized with a T-bet immunogen as described above, and the culture supernatants of the resulting hybridoma cells are screened to identify a hybridoma producing a monoclonal antibody that binds specifically to T-bet.

Any of the many well known protocols used for fusing lymphocytes and immortalized cell lines can be applied for the purpose of generating an anti-T-bet
25 monoclonal antibody (see, e.g., G. Galfre *et al.* (1977) *Nature* 266:55052; Gefter *et al.* *Somatic Cell Genet.*, cited *supra*; Lerner, *Yale J. Biol. Med.*, cited *supra*; Kenneth, *Monoclonal Antibodies*, cited *supra*). Moreover, the ordinary skilled worker will appreciate that there are many variations of such methods which also would be useful. Typically, the immortal cell line (e.g., a myeloma cell line) is derived from the same
30 mammalian species as the lymphocytes. For example, murine hybridomas can be made by fusing lymphocytes from a mouse immunized with an immunogenic preparation of

the present invention with an immortalized mouse cell line. Preferred immortal cell lines are mouse myeloma cell lines that are sensitive to culture medium containing hypoxanthine, aminopterin and thymidine ("HAT medium"). Any of a number of myeloma cell lines may be used as a fusion partner according to standard techniques, 5 e.g., the P3-NS1/1-Ag4-1, P3-x63-Ag8.653 or Sp2/O-Ag14 myeloma lines. These myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Md. Typically, HAT-sensitive mouse myeloma cells are fused to mouse splenocytes using polyethylene glycol ("PEG"). Hybridoma cells resulting from the fusion are then selected using HAT medium, which kills unfused and unproductively 10 fused myeloma cells (unfused splenocytes die after several days because they are not transformed). Hybridoma cells producing a monoclonal antibody of the invention are detected by screening the hybridoma culture supernatants for antibodies that bind T-bet, e.g., using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a 15 monoclonal anti-T-bet antibody can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g., an antibody phage display library) with T-bet to thereby isolate immunoglobulin library members that bind T-bet. Kits for generating and screening phage display libraries are commercially available (e.g., the Pharmacia *Recombinant Phage Antibody System*, Catalog No. 27-9400-01; and 20 the Stratagene *SurfZAP™ Phage Display Kit*, Catalog No. 240612). Additionally, examples of methods and reagents particularly amenable for use in generating and screening antibody display library can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* International Publication No. WO 92/18619; Dower *et al.* International Publication No. WO 91/17271; Winter *et al.* International Publication 25 WO 92/20791; Markland *et al.* International Publication No. WO 92/15679; Breitling *et al.* International Publication WO 93/01288; McCafferty *et al.* International Publication No. WO 92/01047; Garrard *et al.* International Publication No. WO 92/09690; Ladner *et al.* International Publication No. WO 90/02809; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Husse *et al.* (1989) 30 *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clarkson *et al.* (1991) *Nature* 352:624-628; Gram *et al.*

(1992) *PNAS* 89:3576-3580; Garrad *et al.* (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; Barbas *et al.* (1991) *PNAS* 88:7978-7982; and McCafferty *et al.* *Nature* (1990) 348:552-554.

Additionally, recombinant anti-T-bet antibodies, such as chimeric and

5 humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al.* International Patent Publication PCT/US86/02269; Akira, *et al.*

10 European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al.* European Patent Application 173,494; Neuberger *et al.* PCT Application WO 86/01533; Cabilly *et al.* U.S. Patent No. 4,816,567; Cabilly *et al.* European Patent Application 125,023; Better *et al.* (1988) *Science* 240:1041-1043; Liu *et al.* (1987) *PNAS* 84:3439-3443; Liu *et al.* (1987) *J. Immunol.* 139:3521-3526; Sun *et al.* (1987) *PNAS* 84:214-218; Nishimura *et al.* (1987) *Canc. Res.* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; and Shaw *et al.* (1988) *J. Natl Cancer Inst.* 80:1553-1559; Morrison, S. L. (1985) *Science* 229:1202-1207; Oi *et al.* (1986) *BioTechniques* 4:214; Winter U.S. Patent 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeven *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J. Immunol.* 141:4053-4060.

An anti-T-bet antibody (e.g., monoclonal antibody) can be used to isolate T-bet by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-T-bet antibody can facilitate the purification of natural T-bet from cells and of recombinantly produced T-bet expressed in host cells. Moreover, an anti-T-bet antibody

25 can be used to detect T-bet protein (e.g., in a cellular lysate or cell supernatant). Detection may be facilitated by coupling (i.e., physically linking) the antibody to a detectable substance. Accordingly, in one embodiment, an anti-T-bet antibody of the invention is labeled with a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials

30 and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of

suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and 5 examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S or ^3H .

Yet another aspect of the invention pertains to anti-T-bet antibodies that are obtainable by a process comprising:

- (a) immunizing an animal with an immunogenic T-bet protein, or an immunogenic portion thereof unique to T-bet protein; and
- 10 (b) isolating from the animal antibodies that specifically bind to a T-bet protein.

Methods for immunization and recovery of the specific anti-T-bet antibodies are described further above.

IV. Pharmaceutical Compositions

15 T-bet modulators of the invention (e.g., T-bet inhibitory or stimulatory agents, including T-bet nucleic acid molecules, proteins, antibodies, or compounds identified as modulators of T-bet activity) can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the modulatory agent and a pharmaceutically acceptable carrier. As used herein the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof 20 in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. For example, solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following 25 components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents;

antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, 5 such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the 10 extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor ELTM (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy 15 syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethyleneglycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the 20 maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium 25 chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of 30 ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle

which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient

5 from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared

10 using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as

15 microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

20 In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid.

25 Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled

30 in the art, for example, as described in U.S. Patent No. 4,522,811.

V. Methods of the Invention

Another aspect of the invention pertains to methods of using the various T-bet compositions of the invention. For example, the invention provides a method for detecting the presence of T-bet activity in a biological sample. The method involves 5 contacting the biological sample with an agent capable of detecting T-bet activity, such as T-bet protein or T-bet mRNA, such that the presence of T-bet activity is detected in the biological sample.

A preferred agent for detecting T-bet mRNA is a labeled nucleic acid probe capable of specifically hybridizing to T-bet mRNA. The nucleic acid probe can be, for 10 example, the T-bet DNA of SEQ ID NO: 1 or 3, such as an oligonucleotide of at least about 500, 600, 800, 900, 1000, 1200, 1400, or 1600 nucleotides in length and which specifically hybridizes under stringent conditions to T-bet mRNA.

A preferred agent for detecting T-bet protein is a labeled antibody capable of binding to T-bet protein. Antibodies can be polyclonal, or more preferably, monoclonal. 15 An intact antibody, or a fragment thereof (e.g., Fab or F(ab')₂) can be used. The term "labeled", with regard to the probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (*i.e.*, physically linking) a detectable substance to the probe or antibody, as well as indirect labeling of the probe or antibody by reactivity with another reagent that is directly labeled. Examples of indirect labeling include 20 detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. The term "biological sample" is intended to include tissues, cells and biological fluids. For example, techniques for detection of T-bet mRNA include Northern hybridizations and *in situ* hybridizations. Techniques for detection of T-bet 25 protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence.

Such assays are useful in detecting syndromes characterized by developmental defects. For example, mutations in the human T-box genes *TBX5* and *TBX3* (orthologs of mouse *Tbx5* and *Tbx3*) are responsible for the autosomal dominant genetic diseases 30 Holt-Oram syndrome and ulnar-mammary syndrome respectively (Bamshad, M., et al. 1997. *Nature Genetics* 16: 311; Basson, C. T., et al. 1997. *Nature Genetics* 15:30; Li,

Q. Y., et al. 1997. *Nature Genetics* 15: 21; Spranger, S., et al. 1997. *J. Med. Genet.* 3:978). These syndromes are characterized by developmental defects and might have been predicted by the patterns of expression of Tbx5 and Tbx3 respectively. Holt-Oram syndrome affects the heart and upper limbs while ulnar-mammary syndrome affects

5 limb, apocrine gland, tooth and genital development. Both syndromes are characterized by developmental defects and might have been predicted by the patterns of expression of Tbx5 and Tbx3 respectively. The mutations in these patients involve only one allele of the T-box gene- thus it has been postulated that haploinsufficiency of Tbx3 and Tbx 5 cause these two diseases. Recently it has been demonstrated that provision of Tbx4 and

10 Tbx5 to developing chick embryos controls limb bud identity (Rodriguez-Esteban et al. 1999; Takeuchi et al. 1999). These discoveries emphasize the critical importance of this family in vertebrate development.

In addition, the existence of T gene homologs in many species provides strong evidence for its function as a transcription factor that regulates a set of as yet unknown

15 target genes involved in mesoderm development. The recent prominence of the T-box family arises from its clear importance in diverse developmental processes, exemplified most dramatically by the T-box mutations in human disease. The generation of mature T cells from thymocyte stem cells and of differentiated Th cells from naive precursors can also be viewed as tightly regulated developmental processes. This discovery that T-bet is

20 responsible for the development of the Th1 lineage demonstrates an important role for this newest T-box family member in the lymphoid system.

The invention further provides methods for identifying compounds that modulate the activity of a T-bet protein. For example, the invention provides a method for identifying a compound that modulates the activity of a T-bet protein, comprising

25 providing an indicator composition that comprises a T-bet protein;

contacting the indicator composition with a test compound; and

determining the effect of the test compound on the activity of the T-bet protein in the indicator composition to thereby identify a compound that modulates the activity of a T-bet protein.

Specific embodiments of the screening methods of the invention exploit the ability of T-bet proteins to bind to DNA (e.g., the ability to bind to an IL-2 or IFN-gamma promoter) and/or to regulate gene expression (e.g., regulate expression of a Th1-associated cytokine gene, e.g., by repressing the IL-2 gene, transactivate the IFN- γ gene) 5 and/or to redirect polarized Th2 cells into the Th1 pathway.

In a preferred embodiment of the screening assays of the invention, the indicator composition comprises an indicator cell, wherein said indicator cell comprises: (i) the a T-bet protein and (ii) a reporter gene responsive to the T-bet protein. Preferably, the indicator cell contains:

- 10 i) a recombinant expression vector encoding the T-bet; and
- ii) a vector comprising regulatory sequences of a Th1-associated cytokine gene operatively linked a reporter gene; and said method comprising:
 - a) contacting the indicator cell with a test compound;
 - b) determining the level of expression of the reporter gene in the indicator cell in 15 the presence of the test compound; and
 - c) comparing the level of expression of the reporter gene in the indicator cell in the presence of the test compound with the level of expression of the reporter gene in the indicator cell in the absence of the test compound to thereby identify a compound that modulates the activity of T-bet.
- 20 In another preferred embodiment, the indicator composition comprises a preparation of: (i) a T-bet protein and (ii) a DNA molecule to which the T-bet binds, and said method comprising:
 - a) contacting the indicator composition with a test compound;
 - b) determining the degree of interaction of the T-bet protein and the DNA 25 molecule in the presence of the test compound; and
 - c) comparing the degree of interaction of the T-bet and the DNA molecule in the presence of the test compound with the degree of interaction of the T-bet protein and the DNA molecule in the absence of the test compound to thereby identify a compound that modulates the activity of T-bet.
- 30 Preferably, the DNA molecule to which T-bet binds comprises a T-box binding sequence.

In another preferred embodiment, the method identifies proteins that interact with T-bet. In this embodiment,

the indicator composition is an indicator cell, which indicator cell comprises:

- 5 i) a reporter gene operably linked to a transcriptional regulatory sequence; and
- ii) a first chimeric gene which encodes a first fusion protein, said first fusion protein including T-bet;

the test compound comprises a library of second chimeric genes, which library encodes second fusion proteins;

10 expression of the reporter gene being sensitive to interactions between the first fusion protein, the second fusion protein and the transcriptional regulatory sequence; and wherein the effect of the test compound on T-bet in the indicator composition is determined by determining the level of expression of the reporter gene in the indicator cell to thereby identify a test compound comprising a protein that interacts with T-bet.

15 In a preferred embodiment, the library of second chimeric genes is prepared from cDNA library from Th2 cells.

In a preferred embodiment of the screening assays of the invention, once a test compound is identified as modulating the activity of T-bet, the effect of the test compound on an immune response is then tested. Accordingly, the screening methods 20 of the invention can further comprise determining the effect of the compound on an immune response to thereby identify a compound that modulates an immune response. In one embodiment, the effect of the compound on an immune response is determined by determining the effect of the compound on expression of a Th1-associated cytokine gene, such as an interferon-gamma gene. As used herein, the term "Th1-associated 25 cytokine" is intended to refer to a cytokine that is produced preferentially or exclusively by Th1 cells rather than by Th2 cells. Examples of Th1-associated cytokines include IFN-gamma, IL-2, TNF, and lymphotoxin (LT). In another embodiment, the effect of the compound of interest on an immune response is determined by determining the effect of the compound on development of T helper type 1 (Th1) or T helper type 2 (Th2) cells.

Recombinant expression vectors that can be used for expression of T-bet in the indicator cell are known in the art (see discussions above). In one embodiment, within the expression vector the T-bet-coding sequences are operatively linked to regulatory sequences that allow for constitutive expression of T-bet in the indicator cell (e.g., viral

5 regulatory sequences, such as a cytomegalovirus promoter/enhancer, can be used). Use of a recombinant expression vector that allows for constitutive expression of T-bet in the indicator cell is preferred for identification of compounds that enhance or inhibit the activity of T-bet. In an alternative embodiment, within the expression vector the T-bet-coding sequences are operatively linked to regulatory sequences of the endogenous T-

10 bet gene (*i.e.*, the promoter regulatory region derived from the endogenous T-bet gene). Use of a recombinant expression vector in which T-bet expression is controlled by the endogenous regulatory sequences is preferred for identification of compounds that enhance or inhibit the transcriptional expression of T-bet.

In methods in which a Th1-associated cytokine gene is utilized (e.g., as a reporter gene), preferably, the Th1-associated cytokine is interferon-gamma or IL-2. For example, the IL-2 promoter reveals a T-box binding site at -240 to -220 just 5' of the NF κ B site. As described in the appended examples, T-bet was isolated in a yeast one hybrid screening assay based on its ability to bind to the IL-2 promoter. Accordingly, in one embodiment, a method of the invention utilizes a reporter gene construct containing

20 this region of the proximal IL-2 promoter, most preferably nucleotides -240 to -220 of the IL-2 promoter.

A variety of reporter genes are known in the art and are suitable for use in the screening assays of the invention. Examples of suitable reporter genes include those which encode chloramphenicol acetyltransferase, beta-galactosidase, alkaline

25 phosphatase or luciferase. Standard methods for measuring the activity of these gene products are known in the art.

A variety of cell types are suitable for use as an indicator cell in the screening assay. Preferably a cell line is used which does not normally express T-bet, such as a B cell or a Th2 cell clone. Nonlymphoid cell lines can also be used as indicator cells, such

30 as the HepG2 hepatoma cell line. Yeast cells also can be used as indicator cells.

In one embodiment, the level of expression of the reporter gene in the indicator cell in the presence of the test compound is higher than the level of expression of the reporter gene in the indicator cell in the absence of the test compound and the test compound is identified as a compound that stimulates the expression or activity of T-bet.

5 In another embodiment, the level of expression of the reporter gene in the indicator cell in the presence of the test compound is lower than the level of expression of the reporter gene in the indicator cell in the absence of the test compound and the test compound is identified as a compound that inhibits the expression or activity of T-bet.

Alternative to the use of a reporter gene construct, compounds that modulate the expression or activity of T-bet can be identified by using other "read-outs." For example, an indicator cell can be transfected with a T-bet expression vector, incubated in the presence and in the absence of a test compound, and Th1-associated cytokine production can be assessed by detecting cytokine mRNA (e.g., interferon-gamma mRNA) in the indicator cell or cytokine secretion (i.e., interferon-gamma) into the culture supernatant. Standard methods for detecting cytokine mRNA, such as reverse transcription-polymerase chain reaction (RT-PCR) are known in the art. Standard methods for detecting cytokine protein in culture supernatants, such as enzyme linked immunosorbent assays (ELISA) are also known in the art.

As described above, the invention provides a screening assay for identifying 20 proteins (e.g., proteins in Th1 cells) that interact with T-bet.

In one embodiment, such assays can be designed based on the two-hybrid assay system (also referred to as an interaction trap assay) known in the art (see e.g., Field U.S. Patent No. 5.283.173; Zervos *et al.* (1993) *Cell* 72:223-232; Madura *et al.* (1993) *J. Biol. Chem.* 268:12046-12054; Bartel *et al.* (1993) *Biotechniques* 14:920-924; and 25 Iwabuchi *et al.* (1993) *Oncogene* 8:1693-1696). The two-hybrid assay is generally used for identifying proteins that interact with a particular target protein. The assay employs gene fusions to identify proteins capable of interacting to reconstitute a functional transcriptional activator. The transcriptional activator consists of a DNA-binding domain and a transcriptional activation domain, wherein both domains are required to 30 activate transcription of genes downstream from a target sequence (such as an upstream activator sequence (UAS) for GAL4). DNA sequences encoding a target "bait" protein

are fused to either of these domains and a library of DNA sequences is fused to the other domain. "Fish" fusion proteins (generated from the fusion library) capable of binding to the target-fusion protein (e.g., a target GAL4-fusion "bait") will generally bring the two domains (DNA-binding domain and transcriptional activation domain) into close enough proximity to activate the transcription of a reporter gene inserted downstream from the target sequence. Thus, the "fish" proteins can be identified by their ability to reconstitute a functional transcriptional activator (e.g., a functional GAL4 transactivator).

This general two-hybrid system can be applied to the identification of proteins in cells (e.g., Th1 cells) that interact with T-bet by construction of a target T-bet fusion protein (e.g., a T-bet/GAL4 binding domain fusion as the "bait") and a cDNA library of "fish" fusion proteins (e.g., a cDNA/GAL4 activation domain library), wherein the cDNA library is prepared from mRNA of a cell type of interest (e.g., Th1 cells), and introducing these constructs into a host cell that also contains a reporter gene construct linked to a regulatory sequence responsive to T-bet (e.g., an IL-2 promoter sequence, for example, as discussed above). Preferably, the transactivation domain(s) of T-bet will be (which have been mapped to both the 5' and 3' ends) will be deleted in the "bait" construct. In a preferred embodiment, the bait construct will include the T-box domain. In one embodiment, at least one site of tyrosine phosphorylation will also be included.

Dominant negative T-bet proteins can also be used to screen for interactors to further localize sites of T-bet which are required for interaction. cDNAs encoding proteins that interact with T-bet can be identified based upon transactivation of the reporter gene construct.

Alternatively, a "single-hybrid" assay, such as that described in Sieweke, M.H. *et al.* (1996) *Cell* **85**:49-60, can be used to identify proteins that interact with T-bet. This assay is a modification of the two-hybrid system discussed above. In this system, the "bait" is a transcription factor from which the transactivation domain has been removed (e.g., T-bet from which a transactivation domain has been removed) and the "fish" is a non-fusion cDNA library (e.g., a cDNA library prepared from Th1 cells). These constructs are introduced into host cells (e.g., yeast cells) that also contains a reporter gene construct linked to a regulatory sequence responsive to T-bet (e.g., comprising a T-

box binding a region, such as a region of the IL-2 promoter responsive to T-bet). cDNAs encoding proteins that interact with T-bet can be identified based upon transactivation of the reporter gene construct.

In another embodiment, representational difference analysis (RDA) and

5 microchip DNA array analysis to isolate T-bet target genes. For example, differential display or subtraction methods coupled with PCR (RDA; see e.g., Hubank, M. & Schatz, D. G. 1994. *Nuc. Acid Res.* **22**, 5640-5648; Chang, Y., et al. 1994. *Science* **266**, 1865; von Stein, O. D., et al. 1997. *Nuc. Acid Res.* **25**, 2598; Lisitsyn, N. & Wigler, M. 1993. *Science* **259**, 946) employing subtracted or unsubtracted probes or, most recently, DNA

10 microchip array hybridization (Welford et al. 1998. *Nucl. Acids. Res.* **15**:3059) can be used. In performing such assays, a variety of cells can be used, e.g., normal cells, cells engineered to express T-bet, or cells from mice lacking T-bet or overexpressing T-bet (e.g., from a transgenic non-human animal) can be used.

As described above, the invention provides a screening assay for identifying

15 compounds that modulate the interaction of T-bet and a T-box binding region (e.g., an IL-2 gene regulatory region). Assays are known in the art that detect the interaction of a DNA binding protein with a target DNA sequence (e.g., electrophoretic mobility shift assays, DNase I footprinting assays and the like). By performing such assays in the presence and absence of test compounds, these assays can be used to identify

20 compounds that modulate (e.g., inhibit or enhance) the interaction of the DNA binding protein with its target DNA sequence.

In one embodiment, the amount of binding of T-bet to the DNA fragment in the presence of the test compound is greater than the amount of binding of T-bet to the DNA fragment in the absence of the test compound, in which case the test compound is

25 identified as a compound that enhances binding of T-bet. In another embodiment, the amount of binding of T-bet to the DNA fragment in the presence of the test compound is less than the amount of binding of T-bet to the DNA fragment in the absence of the test compound, in which case the test compound is identified as a compound that inhibits binding of T-bet.

Yet another aspect of the invention pertains to methods of modulating T-bet activity in a cell. The modulatory methods of the invention involve contacting the cell with an agent that modulates T-bet activity such that T-bet activity in the cell is modulated. The agent may act by modulating the activity of T-bet protein in the cell or 5 by modulating transcription of the T-bet gene or translation of the T-bet mRNA. As used herein, the term "modulating" is intended to include inhibiting or decreasing T-bet activity and stimulating or increasing T-bet activity. Accordingly, in one embodiment, the agent inhibits T-bet activity. In another embodiment, the agent stimulates T-bet activity.

10 In yet another aspect, the invention provides a method for modulating amount of T helper-type 2 and or T helper-type 1 cytokines by a cell. The method involves contacting a cell with an agent that modulates the activity of T-bet. For example, agents which stimulate T-bet activity upregulate the Th1 cytokine IFN-gamma, while these same agents down regulate the Th2 cytokine IL-4.

15 In a further aspect, the invention provides a method for modulating the pattern of cytokines which is produced by a cell. The method involves contacting a cell with an agent that modulates the activity of T-bet. For example, agents which stimulate T-bet activity can induce IFN-gamma production in a cell which does not normally produce IFN-gamma and can repress IL-4 production; such agents can be used, e.g., to redirect 20 the cytokine secretion profile of a Th2 cell to that of a Th1 cell.

A. Inhibitory Agents

According to a modulatory method of the invention, T-bet activity is inhibited in a cell by contacting the cell with an inhibitory agent. Inhibitory agents of the invention 25 can be, for example, intracellular binding molecules that act to inhibit the expression or activity of T-bet. As used herein, the term "intracellular binding molecule" is intended to include molecules that act intracellularly to inhibit the expression or activity of a protein by binding to the protein itself, to a nucleic acid (e.g., an mRNA molecule) that encodes the protein or to a target with which the protein normally interacts (e.g., to a 30 DNA target sequence to which T-bet binds). Examples of intracellular binding molecules, described in further detail below, include antisense T-bet nucleic acid

molecules (e.g., to inhibit translation of T-bet mRNA), intracellular anti-T-bet antibodies (e.g., to inhibit the activity of T-bet protein) and dominant negative mutants of the T-bet protein.

In one embodiment, an inhibitory agent of the invention is an antisense nucleic acid molecule that is complementary to a gene encoding T-bet or to a portion of said gene, or a recombinant expression vector encoding said antisense nucleic acid molecule. The use of antisense nucleic acids to downregulate the expression of a particular protein in a cell is well known in the art (see e.g., Weintraub, H. *et al.*, Antisense RNA as a molecular tool for genetic analysis. *Reviews - Trends in Genetics*, Vol. 1(1) 1986;

10 Askari, F.K. and McDonnell, W.M. (1996) *N. Eng. J. Med.* 334:316-318; Bennett, M.R. and Schwartz, S.M. (1995) *Circulation* 92:1981-1993; Mercola, D. and Cohen, J.S. (1995) *Cancer Gene Ther.* 2:47-59; Rossi, J.J. (1995) *Br. Med. Bull.* 51:217-225; Wagner, R.W. (1994) *Nature* 372:333-335). An antisense nucleic acid molecule comprises a nucleotide sequence that is complementary to the coding strand of another nucleic acid molecule (e.g., an mRNA sequence) and accordingly is capable of hydrogen bonding to the coding strand of the other nucleic acid molecule. Antisense sequences complementary to a sequence of an mRNA can be complementary to a sequence found in the coding region of the mRNA, the 5' or 3' untranslated region of the mRNA or a region bridging the coding region and an untranslated region (e.g., at the junction of the 15 5' untranslated region and the coding region). Furthermore, an antisense nucleic acid can be complementary in sequence to a regulatory region of the gene encoding the mRNA, for instance a transcription initiation sequence or regulatory element. Preferably, an antisense nucleic acid is designed so as to be complementary to a region preceding or spanning the initiation codon on the coding strand or in the 3' untranslated 20 region of an mRNA. An antisense nucleic acid for inhibiting the expression of T-bet protein in a cell can be designed based upon the nucleotide sequence encoding the T-bet protein (e.g., SEQ ID NO: 1 or 3), constructed according to the rules of Watson and Crick base pairing.

25 An antisense nucleic acid can exist in a variety of different forms. For example, the antisense nucleic acid can be an oligonucleotide that is complementary to only a portion of a T-bet gene. An antisense oligonucleotides can be constructed using

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chemical synthesis procedures known in the art. An antisense oligonucleotide can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic

5 acids, *e.g.* phosphorothioate derivatives and acridine substituted nucleotides can be used. To inhibit T-bet expression in cells in culture, one or more antisense oligonucleotides can be added to cells in culture media, typically at about 200 µg oligonucleotide/ml.

Alternatively, an antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense

10 orientation (*i.e.* nucleic acid transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest). Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the expression of the antisense RNA molecule in a cell of interest, for instance promoters and/or enhancers or other regulatory sequences can be chosen which
15 direct constitutive, tissue specific or inducible expression of antisense RNA. For example, for inducible expression of antisense RNA, an inducible eukaryotic regulatory system, such as the Tet system (*e.g.*, as described in Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551; Gossen, M. *et al.* (1995) *Science* 268:1766-1769; PCT Publication No. WO 94/29442; and PCT Publication No. WO 96/01313) can
20 be used. The antisense expression vector is prepared as described above for recombinant expression vectors, except that the cDNA (or portion thereof) is cloned into the vector in the antisense orientation. The antisense expression vector can be in the form of, for example, a recombinant plasmid, phagemid or attenuated virus. The antisense expression vector is introduced into cells using a standard transfection technique, as
25 described above for recombinant expression vectors.

In another embodiment, an antisense nucleic acid for use as an inhibitory agent is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region (for reviews on ribozymes see *e.g.*, Ohkawa, J. *et al.*

30 (1995) *J. Biochem.* 118:251-258; Sigurdsson, S.T. and Eckstein, F. (1995) *Trends Biotechnol.* 13:286-289; Rossi, J.J. (1995) *Trends Biotechnol.* 13:301-306; Kiehn topf,

M. *et al.* (1995) *J. Mol. Med.* 73:65-71). A ribozyme having specificity for T-bet mRNA can be designed based upon the nucleotide sequence of the T-bet cDNA. For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the base sequence of the active site is complementary to the base sequence to be cleaved in a

5 T-bet mRNA. See for example U.S. Patent Nos. 4,987,071 and 5,116,742, both by Cech *et al.* Alternatively, T-bet mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See for example Bartel, D. and Szostak, J.W. (1993) *Science* 261: 1411-1418.

Another type of inhibitory agent that can be used to inhibit the expression and/or 10 activity of T-bet in a cell is an intracellular antibody specific for the T-bet protein. The use of intracellular antibodies to inhibit protein function in a cell is known in the art (see e.g., Carlson, J. R. (1988) *Mol. Cell. Biol.* 8:2638-2646; Biocca, S. *et al.* (1990) *EMBO J.* 9:101-108; Werge, T.M. *et al.* (1990) *FEBS Letters* 274:193-198; Carlson, J.R. (1993) *Proc. Natl. Acad. Sci. USA* 90:7427-7428; Marasco, W.A. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:7889-7893; Biocca, S. *et al.* (1994) *Bio/Technology* 12:396-399; Chen, S-Y. *et al.* (1994) *Human Gene Therapy* 5:595-601; Duan, L *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5075-5079; Chen, S-Y. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:5932-5936; Beerli, R.R. *et al.* (1994) *J. Biol. Chem.* 269:23931-23936; Beerli, R.R. *et al.* (1994) *Biochem. Biophys. Res. Commun.* 204:666-672; Mhashilkar, A.M. *et al.* 15 (1995) *EMBO J.* 14:1542-1551; Richardson, J.H. *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:3137-3141; PCT Publication No. WO 94/02610 by Marasco *et al.*; and PCT Publication No. WO 95/03832 by Duan *et al.*).

To inhibit protein activity using an intracellular antibody, a recombinant 20 expression vector is prepared which encodes the antibody chains in a form such that, upon introduction of the vector into a cell, the antibody chains are expressed as a functional antibody in an intracellular compartment of the cell. For inhibition of T-bet activity according to the inhibitory methods of the invention, an intracellular antibody that specifically binds the T-bet protein is expressed in the cytoplasm of the cell. To 25 prepare an intracellular antibody expression vector, antibody light and heavy chain 30 cDNAs encoding antibody chains specific for the target protein of interest, e.g., T-bet, are isolated, typically from a hybridoma that secretes a monoclonal antibody specific for

the T-bet protein. Hybridomas secreting anti- T-bet monoclonal antibodies, or recombinant anti-T-bet monoclonal antibodies, can be prepared as described above.

Once a monoclonal antibody specific for T-bet protein has been identified (e.g., either a hybridoma-derived monoclonal antibody or a recombinant antibody from a

5 combinatorial library), DNAs encoding the light and heavy chains of the monoclonal antibody are isolated by standard molecular biology techniques. For hybridoma derived antibodies, light and heavy chain cDNAs can be obtained, for example, by PCR amplification or cDNA library screening. For recombinant antibodies, such as from a phage display library, cDNA encoding the light and heavy chains can be recovered from 10 the display package (e.g., phage) isolated during the library screening process.

Nucleotide sequences of antibody light and heavy chain genes from which PCR primers or cDNA library probes can be prepared are known in the art. For example, many such sequences are disclosed in Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services.

15 NIH Publication No. 91-3242 and in the "Vbase" human germline sequence database.

Once obtained, the antibody light and heavy chain sequences are cloned into a recombinant expression vector using standard methods. To allow for cytoplasmic expression of the light and heavy chains, the nucleotide sequences encoding the hydrophobic leaders of the light and heavy chains are removed. An intracellular

20 antibody expression vector can encode an intracellular antibody in one of several different forms. For example, in one embodiment, the vector encodes full-length antibody light and heavy chains such that a full-length antibody is expressed intracellularly. In another embodiment, the vector encodes a full-length light chain but only the VH/CH1 region of the heavy chain such that a Fab fragment is expressed 25 intracellularly. In the most preferred embodiment, the vector encodes a single chain antibody (scFv) wherein the variable regions of the light and heavy chains are linked by a flexible peptide linker (e.g., (Gly₄Ser)₃) and expressed as a single chain molecule. To inhibit T-bet activity in a cell, the expression vector encoding the anti-T-bet intracellular antibody is introduced into the cell by standard transfection methods, as discussed 30 hereinbefore.

Yet another form of an inhibitory agent of the invention is an inhibitory form of T-bet, also referred to herein as a dominant negative inhibitor, e.g., a form of T-bet in which the tyrosine phosphorylation sites have been mutated or, for example, a mutated form of T-bet in which the transactivation domain has been removed. Such dominant negative T-bet proteins can be expressed in cells using a recombinant expression vector encoding the T-bet protein, which is introduced into the cell by standard transfection methods. To express a mutant form of T-bet lacking tyrosine phosphorylation sites or a transactivation domain, nucleotide sequences encoding a transactivation domain of T-bet are mutated or removed from the *T-bet* coding sequences by standard recombinant DNA techniques. The truncated DNA is inserted into a recombinant expression vector, which is then introduced into a cell to allow for expression of the altered form of T-bet, in the cell.

Other inhibitory agents that can be used to inhibit the activity of a T-bet protein are chemical compounds that directly inhibit T-bet activity or inhibit the interaction between T-bet and target DNA or another protein. Such compounds can be identified using screening assays that select for such compounds, as described in detail above.

B. Stimulatory Agents

According to a modulatory method of the invention, T-bet activity is stimulated in a cell by contacting the cell with a stimulatory agent. Examples of such stimulatory agents include active T-bet protein and nucleic acid molecules encoding T-bet that are introduced into the cell to increase T-bet activity in the cell. A preferred stimulatory agent is a nucleic acid molecule encoding a T-bet protein, wherein the nucleic acid molecule is introduced into the cell in a form suitable for expression of the active T-bet protein in the cell. To express a T-bet protein in a cell, typically a T-bet-encoding DNA is first introduced into a recombinant expression vector using standard molecular biology techniques, as described herein. A T-bet-encoding DNA can be obtained, for example, by amplification using the polymerase chain reaction (PCR), using primers based on the T-bet nucleotide sequence. Following isolation or amplification of T-bet-encoding DNA, the DNA fragment is introduced into an expression vector and transfected into target cells by standard methods, as described herein.

Other stimulatory agents that can be used to stimulate the activity of a T-bet protein are chemical compounds that stimulate T-bet activity in cells, such as compounds that directly stimulate T-bet protein and compounds that promote the interaction between T-bet and target DNA or other proteins. Such compounds can be

5 identified using screening assays that select for such compounds, as described in detail above.

The modulatory methods of the invention can be performed *in vitro* (e.g., by culturing the cell with the agent or by introducing the agent into cells in culture) or, alternatively, *in vivo* (e.g., by administering the agent to a subject or by introducing the agent into cells of a subject, such as by gene therapy). For practicing the modulatory method *in vitro*, cells can be obtained from a subject by standard methods and incubated (*i.e.*, cultured) *in vitro* with a modulatory agent of the invention to modulate T-bet activity in the cells. For example, peripheral blood mononuclear cells (PBMCs) can be obtained from a subject and isolated by density gradient centrifugation, *e.g.*, with

10 Ficoll/Hypaque. Specific cell populations can be depleted or enriched using standard methods. For example, T cells can be enriched for example, by positive selection using antibodies to T cell surface markers, for example by incubating cells with a specific primary monoclonal antibody (mAb), followed by isolation of cells that bind the mAb using magnetic beads coated with a secondary antibody that binds the primary mAb.

15 Specific cell populations can also be isolated by fluorescence activated cell sorting according to standard methods. If desired, cells treated *in vitro* with a modulatory agent of the invention can be readministered to the subject. For administration to a subject, it may be preferable to first remove residual agents in the culture from the cells before administering them to the subject. This can be done for example by a Ficoll/Hypaque

20 gradient centrifugation of the cells. For further discussion of *ex vivo* genetic modification of cells followed by readministration to a subject, see also U.S. Patent No. 5,399,346 by W.F. Anderson *et al.*

For practicing the modulatory method *in vivo* in a subject, the modulatory agent can be administered to the subject such that T-bet activity in cells of the subject is modulated. The term "subject" is intended to include living organisms in which an

immune response can be elicited. Preferred subjects are mammals. Examples of subjects include humans, monkeys, dogs, cats, mice, rats, cows, horses, goats and sheep.

For stimulatory or inhibitory agents that comprise nucleic acids (including recombinant expression vectors encoding T-bet protein, antisense RNA, intracellular

5 antibodies or dominant negative inhibitors), the agents can be introduced into cells of the subject using methods known in the art for introducing nucleic acid (e.g., DNA) into cells *in vivo*. Examples of such methods encompass both non-viral and viral methods, including:

Direct Injection: Naked DNA can be introduced into cells *in vivo* by directly 10 injecting the DNA into the cells (see e.g., Acsadi *et al.* (1991) *Nature* 332:815-818; Wolff *et al.* (1990) *Science* 247:1465-1468). For example, a delivery apparatus (e.g., a "gene gun") for injecting DNA into cells *in vivo* can be used. Such an apparatus is commercially available (e.g., from BioRad).

Cationic Lipids: Naked DNA can be introduced into cells *in vivo* by complexing 15 the DNA with cationic lipids or encapsulating the DNA in cationic liposomes. Examples of suitable cationic lipid formulations include N-[1-(2,3-dioleyloxy)propyl]N,N,N-triethylammonium chloride (DOTMA) and a 1:1 molar ratio of 1,2-dimyristyloxy-propyl-3-dimethylhydroxyethylammonium bromide (DMRIE) and dioleoyl phosphatidylethanolamine (DOPE) (see e.g., Logan, J.J. *et al.* (1995) *Gene Therapy* 2:38-49; San, H. *et al.* (1993) *Human Gene Therapy* 4:781-788).

Receptor-Mediated DNA Uptake: Naked DNA can also be introduced into cells 20 *in vivo* by complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C.H. (1988) *J. Biol. Chem.* 263:14621; Wilson *et al.* (1992) *J. Biol. Chem.* 267:963-967; and U.S. Patent No. 25 5,166,320). Binding of the DNA-ligand complex to the receptor facilitates uptake of the DNA by receptor-mediated endocytosis. A DNA-ligand complex linked to adenovirus capsids which naturally disrupt endosomes, thereby releasing material into the cytoplasm can be used to avoid degradation of the complex by intracellular lysosomes (see for example Curiel *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:8850; Cristiano *et al.* 30 (1993) *Proc. Natl. Acad. Sci. USA* 90:2122-2126).

Retroviruses: Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). A recombinant retrovirus can be constructed having a nucleotide sequences of interest incorporated into the retroviral genome. Additionally, portions of the retroviral genome

5 can be removed to render the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing

10 Associates, (1989). Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include ψ Crip, ψ Cre, ψ 2 and ψ Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes,

15 myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT

20 Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Retroviral vectors require target cell division in order for the retroviral genome (and foreign nucleic acid inserted into it) to be integrated into the host genome to stably introduce nucleic acid into the cell. Thus, it may be necessary to stimulate replication of the target cell.

Adenoviruses: The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner *et al.* (1988)

BioTechniques 6:616; Rosenfeld *et al.* (1991) *Science* 252:431-434; and Rosenfeld *et al.*

- 5 1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld *et al.* (1992) cited
- 10 *supra*), endothelial cells (Lemarchand *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding
- 15 potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner *et al.* cited *supra*; Haj-Ahmad and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral
- 20 vectors currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80 % of the adenoviral genetic material.

Adeno-Associated Viruses: Adeno-associated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review

- 25 see Muzyczka *et al.* *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte *et al.* (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski *et al.* (1989) *J. Virol.* 63:3822-3828; and McLaughlin *et al.* (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300
- 30 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin *et al.* (1985)

Mol. Cell. Biol. 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat *et al.* (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin *et al.* (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford *et al.* (1988) *Mol. Endocrinol.*

5 2:32-39; Tratschin *et al.* (1984) *J. Virol.* 51:611-619; and Flotte *et al.* (1993) *J. Biol. Chem.* 268:3781-3790).

The efficacy of a particular expression vector system and method of introducing nucleic acid into a cell can be assessed by standard approaches routinely used in the art. For example, DNA introduced into a cell can be detected by a filter hybridization 10 technique (e.g., Southern blotting) and RNA produced by transcription of introduced DNA can be detected, for example, by Northern blotting, RNase protection or reverse transcriptase-polymerase chain reaction (RT-PCR). The gene product can be detected by an appropriate assay, for example by immunological detection of a produced protein, such as with a specific antibody, or by a functional assay to detect a functional activity 15 of the gene product.

In a preferred embodiment, a retroviral expression vector encoding T-bet is used to express T-bet protein in cells *in vivo*, to thereby stimulate T-bet protein activity *in vivo*. Such retroviral vectors can be prepared according to standard methods known in the art (discussed further above).

20 A modulatory agent, such as a chemical compound, can be administered to a subject as a pharmaceutical composition. Such compositions typically comprise the modulatory agent and a pharmaceutically acceptable carrier. As used herein the term "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption 25 delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions. Pharmaceutical 30 compositions can be prepared as described above in subsection IV.

The identification of T-bet as a key regulator of the development of Th1 cells described herein, and in the repression of the Th2 phenotype, allows for selective manipulation of T cell subsets in a variety of clinical situations using the modulatory methods of the invention. The stimulatory methods of the invention (*i.e.*, methods that 5 use a stimulatory agent to enhance T-bet activity) result in production of IFN-gamma, with concomitant promotion of a Th1 response and downregulation of both IL-2 and IL-4, thus downmodulating the Th2 response. In contrast, the inhibitory methods of the invention (*i.e.*, methods that use an inhibitory agent to downmodulate T-bet activity) inhibit the production of IFN-gamma, with concomitant downregulation of a Th1 10 response and promotion of a Th2 response. Thus, to treat a disease condition wherein a Th1 response is beneficial, a stimulatory method of the invention is selected such that Th1 responses are promoted while downregulating Th2 responses. Alternatively, to treat a disease condition wherein a Th2 response is beneficial, an inhibitory method of the invention is selected such that Th1 responses are downregulated while promoting Th2 15 responses. Application of the methods of the invention to the treatment of disease conditions may result in cure of the condition, a decrease in the type or number of symptoms associated with the condition, either in the long term or short term (*i.e.*, amelioration of the condition) or simply a transient beneficial effect to the subject.

Numerous disease conditions associated with a predominant Th1 or Th2-type 20 response have been identified and could benefit from modulation of the type of response mounted in the individual suffering from the disease condition. Application of the immunomodulatory methods of the invention to such diseases is described in further detail below.

25 A. *Allergies*

Allergies are mediated through IgE antibodies whose production is regulated by the activity of Th2 cells and the cytokines produced thereby. In allergic reactions, IL-4 is produced by Th2 cells, which further stimulates production of IgE antibodies and activation of cells that mediate allergic reactions, *i.e.*, mast cells and basophils. IL-4 30 also plays an important role in eosinophil mediated inflammatory reactions. Accordingly, the stimulatory methods of the invention can be used to inhibit the

production of Th2-associated cytokines, and in particular IL-4, in allergic patients as a means to downregulate production of pathogenic IgE antibodies. A stimulatory agent may be directly administered to the subject or cells (e.g., Thp cells or Th2 cells) may be obtained from the subject, contacted with a stimulatory agent *ex vivo*, and

5 5 readministered to the subject. Moreover, in certain situations it may be beneficial to coadminister to the subject the allergen together with the stimulatory agent or cells treated with the stimulatory agent to inhibit (e.g., desensitize) the allergen-specific response. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines

10 10 (e.g., anti-IL-4 antibodies), to the allergic subject in amounts sufficient to further stimulate a Th1-type response.

B. Cancer

The expression of Th2-promoting cytokines has been reported to be elevated in

15 15 cancer patients (see e.g., Yamamura, M., *et al.* (1993) *J. Clin. Invest.* 91:1005-1010; Pisa, P., *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7708-7712) and malignant disease is often associated with a shift from Th1 type responses to Th2 type responses along with a worsening of the course of the disease. Accordingly, the stimulatory methods of the invention can be used to inhibit the production of Th2-associated cytokines in cancer

20 20 patients, as a means to counteract the Th1 to Th2 shift and thereby promote an ongoing Th1 response in the patients to ameliorate the course of the disease. The stimulatory method can involve either direct administration of an stimulatory agent to a subject with cancer or *ex vivo* treatment of cells obtained from the subject (e.g., Thp or Th2 cells) with a stimulatory agent followed by readministration of the cells to the subject. The

25 25 treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (e.g., anti-IL-4 antibodies), to the recipient in amounts sufficient to further stimulate a Th1-type response.

25 C. *Infectious Diseases*

The expression of Th2-promoting cytokines also has been reported to increase during a variety of infectious diseases, including HIV infection, tuberculosis, leishmaniasis, schistosomiasis, filarial nematode infection and intestinal nematode infection (see e.g.: Shearer, G.M. and Clerici, M. (1992) *Prog. Chem. Immunol.* 54:21-43; Clerici, M and Shearer, G.M. (1993) *Immunology Today* 14:107-111; Fauci, A.S. (1988) *Science* 239:617-623; Locksley, R. M. and Scott, P. (1992) *Immunoparasitology Today* 1:A58-A61; Pearce, E.J., et al. (1991) *J. Exp. Med.* 173:159-166; Grzych, J-M., et al. (1991) *J. Immunol.* 141:1322-1327; Kullberg, M.C., et al. (1992) *J. Immunol.* 148:3264-3270; Bancroft, A.J., et al. (1993) *J. Immunol.* 150:1395-1402; Pearlman, E., et al. (1993) *Infect. Immun.* 61:1105-1112; Else, K.J., et al. (1994) *J. Exp. Med.* 179:347-351) and such infectious diseases are also associated with a Th1 to Th2 shift in the immune response. Accordingly, the stimulatory methods of the invention can be used to inhibit the production of Th2-associated cytokines in subjects with infectious diseases, as a means to counteract the Th1 to Th2 shift and thereby promote an ongoing Th1 response in the patients to ameliorate the course of the infection. The stimulatory method can involve either direct administration of an inhibitory agent to a subject with an infectious disease or *ex vivo* treatment of cells obtained from the subject (e.g., Thp or Th2 cells) with a stimulatory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (e.g., anti-IL-4 antibodies), to the recipient in amounts sufficient to further stimulate a Th1-type response.

25 D. *Autoimmune Diseases*

The inhibitory methods of the invention can be used therapeutically in the treatment of autoimmune diseases that are associated with a Th2-type dysfunction. Many autoimmune disorders are the result of inappropriate activation of T cells that are reactive against self tissue and that promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Modulation of T helper-type responses can have an effect on the course of the autoimmune disease. For example, in

experimental allergic encephalomyelitis (EAE), stimulation of a Th2-type response by administration of IL-4 at the time of the induction of the disease diminishes the intensity of the autoimmune disease (Paul, W.E., *et al.* (1994) *Cell* 76:241-251). Furthermore, recovery of the animals from the disease has been shown to be associated with an

5 increase in a Th2-type response as evidenced by an increase of Th2-specific cytokines (Koury, S. J., *et al.* (1992) *J. Exp. Med.* 176:1355-1364). Moreover, T cells that can suppress EAE secrete Th2-specific cytokines (Chen, C., *et al.* (1994) *Immunity* 1:147-154). Since stimulation of a Th2-type response in EAE has a protective effect against the disease, stimulation of a Th2 response in subjects with multiple sclerosis (for which

10 EAE is a model) is likely to be beneficial therapeutically. The inhibitory methods of the invention can be used to effect such a decrease.

Similarly, stimulation of a Th2-type response in type I diabetes in mice provides a protective effect against the disease. Indeed, treatment of NOD mice with IL-4 (which promotes a Th2 response) prevents or delays onset of type I diabetes that normally

15 develops in these mice (Rapoport, M.J., *et al.* (1993) *J. Exp. Med.* 178:87-99). Thus, stimulation of a Th2 response in a subject suffering from or susceptible to diabetes may ameliorate the effects of the disease or inhibit the onset of the disease.

Yet another autoimmune disease in which stimulation of a Th2-type response may be beneficial is rheumatoid arthritis (RA). Studies have shown that patients with 20 rheumatoid arthritis have predominantly Th1 cells in synovial tissue (Simon, A.K., *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:8562-8566). By stimulating a Th2 response in a subject with RA, the detrimental Th1 response can be concomitantly downmodulated to thereby ameliorate the effects of the disease.

Accordingly, the inhibitory methods of the invention can be used to stimulate 25 production of Th2-associated cytokines in subjects suffering from, or susceptible to, an autoimmune disease in which a Th2-type response is beneficial to the course of the disease. The inhibitory method can involve either direct administration of an inhibitory agent to the subject or *ex vivo* treatment of cells obtained from the subject (*e.g.*, Thp, Th1 cells, B cells, non-lymphoid cells) with an inhibitory agent followed by 30 readministration of the cells to the subject. The treatment may be further enhanced by administering other Th2-promoting agents, such as IL-4 itself or antibodies to Th1-

associated cytokines, to the subject in amounts sufficient to further stimulate a Th2-type response.

In contrast to the autoimmune diseases described above in which a Th2 response is desirable, other autoimmune diseases may be ameliorated by a Th1-type response.

5 Such diseases can be treated using a stimulatory agent of the invention (as described above for cancer and infectious diseases). The treatment may be further enhanced by administrating a Th1-promoting cytokine (e.g., IFN- γ) to the subject in amounts sufficient to further stimulate a Th1-type response.

The efficacy of agents for treating autoimmune diseases can be tested in the 10 above described animal models of human diseases (e.g., EAE as a model of multiple sclerosis and the NOD mice as a model for diabetes) or other well characterized animal models of human autoimmune diseases. Such animal models include the *mrl/lpr/lpr* mouse as a model for lupus erythematosus, murine collagen-induced arthritis as a model for rheumatoid arthritis, and murine experimental myasthenia gravis (see Paul ed., 15 *Fundamental Immunology*, Raven Press, New York, 1989, pp. 840-856). A modulatory (i.e., stimulatory or inhibitory) agent of the invention is administered to test animals and the course of the disease in the test animals is then monitored by the standard methods for the particular model being used. Effectiveness of the modulatory agent is evidenced by amelioration of the disease condition in animals treated with the agent as compared to 20 untreated animals (or animals treated with a control agent).

Non-limiting examples of autoimmune diseases and disorders having an autoimmune component that may be treated according to the invention include diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, myasthenia gravis, systemic lupus 25 erythematosus, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren's Syndrome, including keratoconjunctivitis sicca secondary to Sjögren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus 30 erythematosus, scleroderma, vaginitis, proctitis, drug eruptions,

leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis.

5 Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Crohn's disease, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis.

E. *Transplantation*

10 While graft rejection or graft acceptance may not be attributable exclusively to the action of a particular T cell subset (*i.e.*, Th1 or Th2 cells) in the graft recipient (for a discussion see Dallman, M.J. (1995) *Curr. Opin. Immunol.* 7:632-638), numerous studies have implicated a predominant Th2 response in prolonged graft survival or a predominant Th2 response in graft rejection. For example, graft acceptance has been

15 associated with production of a Th2 cytokine pattern and/or graft rejection has been associated with production of a Th1 cytokine pattern (see *e.g.*, Takeuchi, T. *et al.* (1992) *Transplantation* 53:1281-1291; Tzakis, A.G. *et al.* (1994) *J. Pediatr. Surg.* 29:754-756; Thai, N.L. *et al.* (1995) *Transplantation* 59:274-281). Additionally, adoptive transfer of cells having a Th2 cytokine phenotype prolongs skin graft survival (Maeda, H. *et al.* (1994) *Int. Immunol.* 6:855-862) and reduces graft-versus-host disease (Fowler, D.H. *et al.* (1994) *Blood* 84:3540-3549; Fowler, D.H. *et al.* (1994) *Prog. Clin. Biol. Res.* 389:533-540). Still further, administration of IL-4, which promotes Th2 differentiation, prolongs cardiac allograft survival (Levy, A.E. and Alexander, J.W. (1995) *Transplantation* 60:405-406), whereas administration of IL-12 in combination with anti-

20 IL-10 antibodies, which promotes Th1 differentiation, enhances skin allograft rejection (Gorczyński, R.M. *et al.* (1995) *Transplantation* 60:1337-1341).

Accordingly, the inhibitory methods of the invention can be used to stimulate production of Th2-associated cytokines in transplant recipients to prolong survival of the graft. The inhibitory methods can be used both in solid organ transplantation and in

30 bone marrow transplantation (*e.g.*, to inhibit graft-versus-host disease). The inhibitory method can involve either direct administration of an inhibitory agent to the transplant

recipient or *ex vivo* treatment of cells obtained from the subject (e.g., Thp, Th1 cells, B cells, non-lymphoid cells) with an inhibitory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th2-promoting agents, such as IL-4 itself or antibodies to Th1-associated cytokines, to the 5 recipient in amounts sufficient to further inhibit a Th2-type response.

In addition to the foregoing disease situations, the modulatory methods of the invention also are useful for other purposes. For example, the stimulatory methods of the invention (*i.e.* methods using a stimulatory agent) can be used to stimulate production of Th1-promoting cytokines (e.g., interferon-gamma) *in vitro* for commercial 10 production of these cytokines (e.g., cells can be contacted with the stimulatory agent *in vitro* to stimulate interferon-gamma production and the interferon-gamma can be recovered from the culture supernatant, further purified if necessary, and packaged for commercial use).

Furthermore, the modulatory methods of the invention can be applied to 15 vaccinations to promote either a Th1 or a Th2 response to an antigen of interest in a subject. That is, the agents of the invention can serve as adjuvants to direct an immune response to a vaccine either to a Th1 response or a Th2 response. For example, to promote an antibody response to an antigen of interest (*i.e.*, for vaccination purposes), the antigen and an inhibitory agent of the invention can be coadministered to a subject to 20 promote a Th2 response to the antigen in the subject, since Th2 responses provide efficient B cell help and promote IgG1 production. Alternatively, to promote a cellular immune response to an antigen of interest, the antigen and a stimulatory agent of the invention can be coadministered to a subject to promote a Th1 response to the antigen in a subject, since Th1 responses favor the development of cell-mediated immune 25 responses (e.g., delayed hypersensitivity responses). The antigen of interest and the modulatory agent can be formulated together into a single pharmaceutical composition or in separate compositions. In a preferred embodiment, the antigen of interest and the modulatory agent are administered simultaneously to the subject. Alternatively, in certain situations it may be desirable to administer the antigen first and then the 30 modulatory agent or vice versa (for example, in the case of an antigen that naturally evokes a Th1 response, it may be beneficial to first administer the antigen alone to

stimulate a Th1 response and then administer an inhibitory agent, alone or together with a boost of antigen, to shift the immune response to a Th2 response).

This invention is further illustrated by the following example, which should not be construed as limiting. The contents of all references, patents and published patent 5 applications cited throughout this application are hereby incorporated by reference. Additionally, all nucleotide and amino acid sequences deposited in public databases referred to herein are also hereby incorporated by reference.

A nucleic acid molecule comprising a mouse T-bet cDNA cloned into the EcoRI site of the pJG4-5 vector was deposited with the American Type Culture Collection 10 (Manassas, VA) on November 9, 1999 and assigned Deposit Number PTA-930. A nucleic acid molecule comprising a human T-bet cDNA (prepared from RNA from the human Th1 clone ROT-10) cloned into the PCR 2.1-TOPO vector was deposited with the American Type Culture Collection (Manassas, VA) on January 28, 2000 and assigned Deposit Number PTA-1339. Both deposits were made under the provisions of 15 the Budapest Treaty.

EXAMPLES

The following experimental procedures were used in the examples:

20 **Mice, Cell Lines, Cytokines, Antibodies and Plasmids**

BALB/c mice were obtained from Jackson Laboratories, DO11.10 TcR-transgenic mice (Jacobson, N.G., et al. 1995. *J. Exp. Med.* 181, 1755-1762), and MBP TcR-transgenic mice (Lafaille, J.J., 1994. *Cell* 78, 399-408.) have been described. Mice were used at 5-6 weeks of age. Cell lines and primary cells were maintained in complete 25 medium containing RPMI 1640 supplemented with 10% fetal calf serum (HyClone Laboratories), glutamine (2mM), penicillin (50 units/ml), streptomycin (50 μ g/ml), Hepes (100mM) and β -ME (50 μ M). Jurkat is a human Th1 lymphoma, EL4 a mouse Th0 thymoma, NK3.3 a human NK cell line (Ye, J., 1995. *J. Leuko. Biol.* 58, 225-233.; Kornbluth, J., 1982. *J. Immunol.* 129, 2831-2837), YT a human NK cell line 30 (Yodoi, J., 1985. *J. of Immuno.* 134, 1623-1630), AE7 a mouse Th1 clone, D10 a mouse

Th2 clone and M12 is a B cell lymphoma line. Recombinant IL-4 was obtained from DNAX, human rIL-2 was obtained from Chiron Corp., rIL-12 was obtained from Hoffman LaRoche, and rIL-18 was purchased from Peprotech, Inc. Monoclonal anti-IL-12, monoclonal anti-IFN- γ and monoclonal anti-IL-4 (11B11) were also used (Ohara, J. and Paul, W.E. 1985. *Nature* 315, 333-336). Both the T-bet polyclonal antisera, produced in rabbits, and the mAb were raised against full-length recombinant bacterially produced T-bet. The mAb was produced by fusion of spleen cells from mice to the SP2/O-Ag14 myeloma and is of the IgG1 subtype. Expression plasmids included c-Maf (pMex-maf)(Ho, I-C., et al. 1996. *Cell* 85, 973-983.). NFATp (Hodge, M.R., et al. 10 1996. *Immunity* 4, 1-20) and p65, the latter two cloned into the pCDNA vector.

CD4+ T Cell Purification and In Vitro Cultures

CD4+ T cells were purified from lymph nodes (LN) by flow cytometry using PE-conjugated anti CD4 (RM4-4) (Pharmingen) and sorted using FACS (Mo Flo, 15 Becton Dickenson) to 98-99% purity. For in vitro activation 2 x 10⁶/ml CD4+ cells were resuspended in complete medium and activated with plate-bound 1 μ g/ml anti CD3 (2C11) and 2 μ g/ml anti CD28 (Pharmingen) for 3 days in the presence of 100 units/ml IL2. Cells were then split 1:4 in complete medium and cultured for 4 days in the presence of 100 units/ml IL2. On day 7 after primary stimulation, cells were harvested, 20 washed twice and restimulated at 1 x 10⁶ cells/ml with 1 μ g/ml plate-bound anti CD3 for 1, 3 and 6 hours. For Th1 and Th2 differentiation cultures, non-transgenic or DO11.10 LN and spleen cells were pooled, resuspended in 1 x 10⁶ cells/ml complete medium and cultured under Th1 (10mg/ml anti IL4 [11B11], 10ng/ml rIL12) or Th2 (10mg/ml anti IFN γ , 10ng/ml IL4) conditions with 1 μ g/ml plate-bound anti CD3. Cells were split 1:4 25 on day 3 with complete medium + 100u/ml IL2. On day 7, cells were restimulated with 1 μ g/ml anti CD3 for 4 hours and harvested for RNA preparation (Jacobson, N.G., et al.1995). *J. Exp. Med.* 181, 1755-1762). Supernatants were taken at 24 hours to test for cytokines.

Northern and Western Blot Analysis

Total RNA was isolated from resting and stimulated cells using TRIZOL reagent (Gibco/BRL) and 10 μ g of each sample separated on 1.2% agarose 6% formaldehyde gels, transferred onto Genescreen membrane (NEN) in 20X SSC overnight and

5 covalently bound using a UV Stratalinker (Stratagene). Hybridization of blots was carried out at 42°C as described (Hodge, M.R., et al. 1996. *Immunity* 4, 1-20) using the following cDNA probes labeled with 32P: T-bet, γ -actin. Nuclear and cytoplasmic extracts for western blot analysis were prepared from AE7, D10 and NK3.3 cells. Nuclei were isolated as described (Dolmetsch, R.E., et al. 1997. *Nature* 386, 855-858).

10 Extracted proteins were separated by 8% PAGE followed by electrotransfer to nitrocellulose membranes and probed with a mAb specific for T-bet followed by horseradish peroxidase-conjugated goat anti-mouse IgG and enhanced chemiluminescence according to the instructions of the manufacturer (Amersham).

15 **Transient Transfection Assays**

EL4 and Jurkat cells were transfected using a Bio Rad electroporator (280V, 975 μ F) using 5 X 10 6 cells in 0.4 ml RPMI per transfection with 5 μ g reporter plasmid and 5-10 μ g expression plasmid. Luciferase assays were performed after 24 hrs with the luciferase activity in 20% of each sample measured as per instructions (Promega). The

20 IFN- γ reporter-luciferase construct is derived from the plasmid pB9 which contains the entire human IFN-gamma gene (P. Gray and D.V. Goeddel. 1982. *Nature*, 298:859). The pGL2 luciferase gene was inserted into the first exon of pB9. IL-2-promoter-reporter construct The IL-4 promoter reporter construct, IL-4Luc, contains 807 bp upstream of the murine IL-4 gene.

25

Retroviral Constructs and Transduction

The GFP-RV bicistronic vector has been described (Ouyang, W., et al. 1998. *Immunity* 9:745-755) as has the Phoenix-Eco packaging cell line (Kinoshita, S., et al. 1998. *Cell* 95, 595-604). The GFP-RV vector was constructed by inserting the

30 encephalomyocarditis virus internal ribosomal entry sequence (IRES) and the GFP allele

into the MSCV2.2 retroviral vector (Ouyang, W., et al. 1998. *Immunity* 9:745-755) or IL-2-MSCV vector. Both vectors express two cDNAs. T-bet and the cDNA encoding GFP, simultaneously using an IRES to initiate translation of each mRNA separately. Transfection of the packaging cell line and retroviral transductions of primary T cells 5 were performed essentially as described (Ouyang, W., et al. 1998. *Immunity* 9:745-755).

Intracellular cytokine staining and FACS Analysis

Intracellular staining for cytokines was performed as described (Ouyang, W., et al. 1998. *Immunity* 9:745-755). Primary transgenic or non-transgenic T cells that had 10 been infected with retrovirus for various time periods as indicated were restimulated with PMA (50 ng/ml) and ionomycin (1 uM) for 2 hours and 10 ug/ml Brefeldin A added for an additional 2 hours.

Example 1. Cloning of a novel transcription factor, T-bet

15 Since the Th1-specific region of the IL-2 promoter had been well localized (Brombacher, F., et al. 1994. *Int. Immunol.* 6:189-197.; Rooney, J., et al. 1995. *Mol. Cell. Biol.* 15, 6299-6310; Lederer, J.A., et al. 1994. *J. Immunol.* 152, 77-86; Durand, D., et al. 1988. *Mol. Cell. Biol.* 8, 1715-1724; Hoyos, B., et al. 1989. *Science* 244, 457-450), a yeast one hybrid approach using an IL-2 promoter-reporter and a cDNA library 20 made from the OF6 Th1 clone was chosen to identify Th1 specific transcription factors. To validate this approach, the Th2-specific region of the IL-4 promoter was expressed in yeast and demonstrated to be transactivated by the introduction of c-Maf, but not by several other transcription factors (eg NFAT). C-Maf transactivation did not occur when the c-Maf response element (MARE) was mutated. Thus, the yeast one hybrid approach 25 was utilized.

The EGY48 yeast strain was stably integrated with the IL-2 promoter/histidine construct and transformed with a cDNA library made from an anti-CD3 activated Th1 cell clone, OF6. Of 5.6×10^6 clones screened, 488 were positive in primary screening. Of the 210 clones tested during the secondary screen, 72 proved to be specific for the IL-30 2 promoter. To reduce the number of positive clones, we hybridized the yeast clone cDNA with cDNAs that were differentially expressed in Th1 and Th2 cell lines. These

Th1-Th2 and Th2-Th1 cDNAs were made using the Clontech PCR select kit, radiolabeled and initially used in a pilot experiment to screen the 16 most strongly positive yeast clones. Of those 16 clones, 8 were positive with the Th1 (PL17) specific cDNA product probe and not with the Th2 (D10) specific cDNA product probe.

5 Representative difference analysis (RDA; e.g., Lisitsyn. 1993. *Science*. 259:946; O'Neill and Sinclair. 1997. *Nucleic Acids Res.* 25:2681; Hubank and Schatz. 1994. *Nucleic Acids Research*. 22:5640; Welford et al. 1998. *Nucleic Acids Research*. 26:3059) with Th1-Th2 probe on 16 positive clones with control hybridization of the probe to IL-2, IFN-gamma and IL-4 was performed. The specificity of the Th1 and Th2 10 subtracted cDNA probes is demonstrated by their detection of IL-2 and IFN- γ versus IL-4 respectively.

Restriction enzyme analyses and sequencing data revealed that all 8 of the clones were related. They fell into three groupings based on differences in the 5' and 3' untranslated regions, each of these categories representing an independent cDNA 15 molecule. Comparing the sequence of these clones with the NCBI GenBank Sequence Database yielded homology with the T-box family of transcription factors. Figure 1 shown the nucleotide and amino acid sequences of T-bet.

Example 2. T-bet shares a region of homology with the T-box family members T-brain and comesodermin

20 *Brachyury* or *T* is the founding member of a family of transcription factors that share a 200 amino acid DNA-binding domain called the T-box (reviewed in (Smith, J. 1997. *Current Opinion in Genetics & Development* 7, 474-480; Papaioannou, and Silver. 1998. *Bioessay*. 20:9; Meisler, M.H. 1997. *Mammalian Genome* 8, 799-800.). 25 The *Brachyury* (Greek for 'short tail') mutation was first described in 1927 in heterozygous mutant animals who had a short, slightly kinked tail (Herrmann, B.G., 1990. *Nature* 343, 617-622). There are now eight T-box genes in the mouse not including *Brachyury*. These include *Tbx1-6*, *T-brain-1* (*Tbr-1*) and now, *T-bet*, each with a distinct and usually complex expression pattern. The T-box family of 30 transcription factors is defined by homology of family members in the DNA binding domain. The *T-bet* DNA binding domain (residues 138-327 of murine *T-bet*) is most

similar to the T-box domains of murine T-brain and *Xenopus* eomesodermin and thus places T-bet in the *Tbr1* subfamily of the T-box gene family. The human homologue of the murine T-bet protein is approximately 88 % identical to the mouse T-bet. Figure 1A was derived using a Lipman-Pearson protein alignment (with G penalty set at 4 and gap 5 length penalty set at 12. The similarity index was calculated to be 86.6; the gap number2, the gap length5, and the consensus length 535). T-bet shares a region of homology with the T-box family members T-brain and eomesodermin. The murine T-bet DNA binding domain is most similar to the T-box domains of murine T-brain and *Xenopus* eomesodermin. There is approximately 69% amino acid identity between the 10 three T-box regions. T-bet bears no sequence homology to other T-box family members outside of the T-box domain.

Example 3. T-bet binds to and transactivates consensus T-box sites and has functionally important domains that map to both 5' and 3' regions

15 Recombinant T-bet protein binds to consensus T-box sites and to the T-bet site in the IL-2 promoter. and a complex present in nuclear extracts from anti-CD3-stimulated AE7 Th1 cells binds specifically to a consensus (GGGAATTCACACCTAGGTGAAATTCC) T-box oligonucleotide probe. To test for activity of T-bet in T cells, the following experiments were performed. Jurkat Th1 20 cells were contransfected with T-bet and a luciferase reporter construct. Figure 2A shows the basal level (open bars) and the PMA (50ng/ml) plus ionomycin (1uM) induced (closed bars) promoter activity in Jurkat cells of a luciferase reporter construct containing a minimal thymidine kinase (TK) promoter with or without 4 copies of the consensus T-box site. Each reporter construct was co-transfected with empty pCDNA 25 vector or pCDNA containing the full-length T-bet cDNA as indicated in the figure. The data shown are representative of three independent experiments. Figure 2B shows Jurkat cells transiently transfected with the luciferase reporter construct containing the minimal TK promoter and multimerized consensus T-box sites and pCDNA vector containing the indicated regions of the T-bet cDNA diagrammed at the left of the bar graph. Luciferase 30 activity was measured 24 hours post-transfection. The experiment was repeated three times with similar results. The basal level (open bars) and the PMA (50ng/ml) plus

ionomycin (1uM) induced (closed bars) promoter activity obtained demonstrate that T-bet is active in T cells. and that its activity can be further increased upon stimulation.

Example 4. T-bet expression in T cells is restricted to the Th1 subset and regulated by signals transmitted via the TcR

T-bet was isolated from a Th1 cDNA library and a multiple organ Northern blot analysis revealed T-bet transcripts only in lung, thymus and in peripheral lymphoid organs.

Figure 3A shows that T-bet is preferentially expressed in double negative (DN) thymocytes, not in double positive (DP) or single positive (SP) cells. Northern blot analysis of total cellular RNA isolated from Th1 cell clones (AE7 and D1.1) or Th2 clones (D10 and CDC35) that were treated with media or with plate-bound anti-CD3 (2C11) for 6 hours revealed T-bet transcripts only in the Th1 clones. Total cellular RNA was isolated from Th1 cell clones (AE7 and D1.1) or Th2 clones (D10 and CDC35) that were treated with media or with plate-bound anti-CD3 (2C11) for 6 hours. Total RNA was also isolated from M12 (B-cell lymphoma and EL4 (T-cell thymoma) treated with media or with PMA (50ng/ml) and ionomycin (1uM) for 6 hours. Northern blot analysis was performed with 10 ug of total RNA per lane using standard procedures and probed using the full-length T-bet cDNA. T-bet is prefentially expressed in Th1 clones.

Further, the level of T-bet expression was augmented by signals transmitted via the TcR as evidenced by the induction of T-bet transcripts by anti-CD3. T-bet transcripts were not detected in M12, a B-cell lymphoma, in the Th1 lymphoma Jurkat or in EL4, a Th0-cell thymoma either when these cells were treated with media or with PMA (50ng/ml) and ionomycin (1uM) for 6 hours.

To determine protein levels of T-bet in primary T cells, DO11.10 TcR transgenic splenocytes were cultured under Th1 or Th2 polarizing conditions. At 72 hours the cells were expanded 3-fold in fresh medium with 200U/ml IL-2. On day 7 after primary stimulation, nuclear and cytosolic extracts were prepared from resting or PMA/ionomycin activated (1 hr) bulk culture DO11.10 Th1 and Th2 cells. Nuclear extracts were also prepared from resting M12, EL4, Jurkat, NK3.3, and YT cells. As shown in Figure 3C. among the cell lines, T-bet protein was present in YT cells only.

Figure 3C shows T-bet protein is restricted to Th1 cells and NK cells. Western blot analysis was performed on nuclear and cytosolic extracts prepared from resting or PMA/ionomycin activated (1 hr) bulk culture DO11.10 Th1 and Th2 cells as above. Briefly, DO11.10 Tcrtransgenic splenocytes were activated with OVA peptide (323-339)

5 at 3 x 10⁶ cells/ml in the presence of 10 ng/ml IL-12 and 10 ug/ml anti-IL-4 (11B11) to promote Th1 phenotype development, or 10ng/ml IL-4 and 10 ug/ml anti-IFN-gamma to promote Th2 phenotype development. At 72 hours the cells were expanded 3-fold in fresh medium with 200 U/ml IL-2. On day 7 after primary stimulation, nuclear and cytosolic extracts were prepared from resting or PMA/ionomycin acitivate (1hr) bulk

10 culture DO11.10 Th1 and Th2 cells. Nuclear extracts were also prepared from resting M12 cells, EL4, Jurkat, NK3.3, and YT. 30 ug of nuclear and cytosolic extracts were separated by SDS-PAGE (8% gel), transferred to nitrocellulose, and probed with an anti T-bet antisera. In primary T cells, T-bet protein is selectively expressed in T cells driven along a Th1 but not a Th2 pathway, consistent with the Northern blot analysis of T cell

15 clones and primary T cells shown above.

A monoclonal antibody (mAb) specific for T-bet allowed the direct visualization of T-bet protein by FACS analysis. Figure 3D shows that T-bet can be visualised by FACS in activated AE7 Th1 cells. D10 (Th2) or AE7 (Th1) cells were treated with media or PMA (50ng/ml) plus ionomycin (1uM) for 2 hours and 2uM monensin for an additional 3 hours. Cells were washed with PBS, fixed in 4% paraformaldehyde, permeabilized with 0.5% saponin, and stained with media (dashed line) or an IgG1 isotype control antibody (dotted line) or an affinity-purified anti-T-bet monoclonal antibody α 3D10 (solid line) followed by goat anti-mouse IgG1-PE staining. Cells were analyzed by flow cytometry on a FACSCalibur. Mouse monoclonal antibodies were raised against bacterially produced T-bet. T-bet protein was not detectable in D10 cells, was present at low levels in unstimulated AE7 cells and was present at increased levels in stimulated AE7. Taken together, the experiments detailed here demonstrate that in T cells, T-bet is selectively expressed in Th1 cells where its level of expression is regulated by signals stemming from the TcR.

Example 5. T-bet expression correlates with IFN- γ induction in NK and B cells

The Th1-limited expression of T-bet coupled with its isolation by virtue of binding to a T-box site in the IL-2 promoter suggested that T-bet might activate the transcription of the IL-2 gene. However, it was puzzling that two IL-2-producing cell lines, Jurkat and EL4, did not express T-bet, while the NK cell line YT, which produces IFN- γ but not IL-2, did express T-bet. Further, preliminary experiments did not demonstrate transactivation of the IL-2 gene by T-bet, despite the presence of an excellent T-box site in the IL-2 promoter. Other Th1-specific cytokines include IFN- γ , TNF α and LT. The expression of T-bet correlated well with the expression of IFN- γ .

10 Further, a T-box site was found to be present in the third intron of the human IFN- γ gene. This was especially noteworthy since a Th1-specific DNaseI hypersensitivity site had recently been mapped to this region.

To examine the possibility that T-bet controlled the expression of the IFN- γ gene, the expression of T-bet and the expression of IFN- γ in cells other than Th1 cells was measured. IFN- γ is expressed in natural killer (NK) cells at low levels and is induced to high levels upon treatment with IL-2 and IL-12 (Kornbluth, J., et al. 1982. J. Immunol. 129:2831; Ye et al. 1995. J. Leuko. Biol. 58:225). Therefore, the NK3.3 cell line was treated for 24 h with IL-2, IL-12 and IL-2 plus IL-12, lysates prepared and western blot analysis performed with T-bet mAb as above. Figure 4b demonstrates coordinate induction of T-bet protein and secretion of IFN- γ in NK3.3 cells. The NK3.3 cell line was treated for 24 h with reagents, IL-2, IL-12 and IL-2 plus IL-12, known to induce IFN- γ in NK cells, lysates prepared and western blot analysis performed with T-bet mAb as above. ELISA was performed on supernatants harvested from the cells.

25 B cells, which do not produce IFN- γ at baseline, can be driven to produce large amounts of IFN- γ upon treatment with anti-CD40 antibody and a combination of IL-12 and IL-18 (Yoshimoto, T., 1997. Proc. Natl. Acad. Sci. USA 94, 3948-3953). Purified B cells were treated for 72h with anti-CD40 mAb, rIL-12 and rIL-18, RNA isolated and Northern blot performed using the T-bet cDNA as above. Figure 4A shows induction of T-bet mRNA in B cells treated with this combination of reagents, and the induction of 30 IFN- γ transcripts in these cells was confirmed. In conclusion, while neither cell type

expresses T-bet constitutively, both NK3.3 cells and B cells can be induced to do so under conditions which also result in IFN- γ production. Thus, the pattern of expression of T-bet correlates well with the transcription of the IFN- γ gene.

5 **Example 6. T-bet transactivates the IFN- γ gene in Th cells**

Very little is yet known about the regulatory regions of the IFN- γ gene. In particular, the regions of the gene that direct its tissue-specific expression have not been identified in vitro or in vivo. It has been demonstrated that reporter constructs containing 500 bp or 3 kb of upstream sequence are expressed in both Th1 and Th2 cells

10 (Young, H.A., 1994. *J. of Immuno.* **153**, 3603-3610). ATF-2, NF κ B, AP-1 and Stat4 sites in the IFN- γ promoter or introns are thought to be functionally important, but clearly are not responsible for tissue-specific expression (Young, H.A., 1994. *J. of Immuno.* **153**, 3603-3610; Sica, A., 1997. *J. Biol. Chem.* **272**, 30412-30420; Penix, L., 1993. *J. Exp. Med.* **178**, 1483-1496; Penix, L.A., 1996. *J. Biol. Chem.* **271**, 31964-15 31972). Similarly, although Th1-preferential DNaseI hypersensitive sites have been noted both in the first and third introns, the relevant cis elements located in these introns have not been identified (Young, H.A., et al. 1994. *J. of Immuno.* **153**, 3603-3610; Agarwal, S. and Rao, A. 1998. *Immunity* **9**, 765-775). Therefore, a reporter construct containing the entire IFN- γ gene was utilized for these studies. The IFN- γ reporter gene 20 used includes 3 kb of upstream sequence, the entire coding sequence with all three introns, and 1.5 kb of downstream (Xu, X., et al. 1996. *Science* **273**, 794-796).

The activity of a luciferase reporter construct containing 9 kb of the IFN-gamma gene in the Jurkat human Th1 lymphoma and the mouse EL4 Th0 thymoma was tested. Each reporter construct (10ug) was co-transfected with empty pCDNA vector or 25 pCDNA containing the full-length T-bet cDNA, c-Maf, NFATp or p65 (10ug). The constructs also include the -400 to -40 IL-2 and IL-4 promoter luciferase reporters.

The Th0 mouse T cell thymoma EL4, which produces IL-2 and IL-4 but not IFN- γ , was transfected with a T-bet cDNA expression plasmid and the IFN- γ -luciferase reporter (Figure 5). Introduction of the T-bet expression plasmid resulted in 30 (approximately 20-30 fold) transactivation of the IFN- γ gene compared to empty vector

alone. This was in contrast to the absence of transactivation by two other factors, the Th2-specific transcription factor c-Maf and the Th non-selective transcription factor NFAT. Interestingly, although the NF κ B family member, p65, did not transactivate the IFN- γ reporter on its own, cotransfection of T-bet and p65 resulted in a synergistic
5 activation.

Examination of the IL-2 promoter was also made using a region of the promoter known to be Th1-specific (Lederer, J.A., et al. 1994. J. Immunol. 152, 77-86). T-bet repressed the activity of the IL-2 promoter approximately 10 fold. This was especially apparent upon activation of the promoter by PMA and ionomycin. As before,
10 substantial transactivation of the IFN- γ gene was noted. T-bet activity was specific for the IL-2 and IFN- γ genes since no effect on transactivation of an IL-4 promoter (Figure 5) or a TNF- α promoter was present. These data demonstrate that T-bet specifically activates the transcription of the IFN- γ gene, and represses the transcription of the IL-2 gene.
15 To examine endogenous gene expression, EL4 cells were transiently transfected with T-bet or empty vector, and IFN- γ production measured by ELISA 48 hours after stimulation with PMA/ionomycin (Figure 5). Consistent with the transactivation data shown above, ectopic expression of T-bet in EL4 cells led to measurable IFN- γ production while transfection with vector control did not result in detectable IFN- γ .
20

Example 7. Retroviral gene mediated transfer of T-bet into primary Th cells results in increased IFN- γ production

The experiments described above argue strongly for a critical role of T-bet in controlling the transcription of the IFN- γ gene.

25 A bovine collagen-specific Th0 hybrid was transduced with retroviral constructs containing T-bet GFP or GFP only under the control of the TcR inducible IL-2 promoter. Transduced populations were FACS sorted on GFP twice, rested and then stimulated with anti-CD3 and supernatants collected at 60 hours to measure cytokine production by ELISA. (Figure 6). Control retroviral vectors which had no effect
30 included anti-sense T-bet.

To further test whether T-bet is responsible for the tissue-specific expression of IFN- γ , retroviral gene mediated transfer of T-bet into primary T cells, both non-transgenic and TcR transgenic, was performed. Two different bicistronic retroviruses expressing both T-bet and GFP were used. The first expresses T-bet under the control of 5 an IL-2 inducible promoter, and the second expresses T-bet under control of an MSCV LTR. Similar results were obtained with both constructs.

BALB/c CD4 T cells were infected after 36 hours of primary activation by anti-CD3 plus anti-CD28, harvested on day 7 and intracellular IFN-gamma and IL-2 staining performed 5 hours after stimulation with PMA and ionomycin as described in 10 Experimental Procedures. Data are shown as two-color plots showing GFP expression (FL1) versus intracellular cytokine (FL2) of events gated on expression of CD4. Primary T cells from MBP TcR transgenic mice were stimulated using MBP (Ac1-11) at 6 uM and infection performed on day 1 with IL-2/GFP and IL-2/T-bet/GFP. On day 7, 15 cells were sorted for GFP expression, rested for 1 day and then intracellular cytokine analysis performed after a 5 hour stimulation with PMA and ionomycin.

Naive MBP-transgenic or non-transgenic BALB/c CD4 T cells were activated with MBP 1-11 and anti-CD3 under non-polarizing conditions and were infected with retrovirus on day 1 after primary activation as described (Ouyang, W., et al. 1998. Immunity 9:745-755.). Cells were cultured for 7 days and then GFP expression 20 measured to determine percentage of cells infected. GFP positive cells were sorted and cytokine production measured by intracellular staining after an additional 4 hours stimulation with PMA plus ionomycin.

Transduction of both MBP-TcR transgenic and non-transgenic T cells with T-bet resulted both in an impressive increase in the number of cells producing IFN- γ and in the 25 amount of IFN- γ produced per cell as compared to cells transduced with GFP alone. (Figure 7).

Naive Thp cells, early after stimulation, produce large amounts of IL-2, which is then gradually replaced in polarized Th cells by the effector cytokines IFN- γ and IL-4. Polarized Th1 cells do continue to produce IL-2 but at amounts considerably less than 30 naïve Thp. Polarized Th2 cells shut off the production of IL-2. T-bet transduced Th cells

produced somewhat less IL-2 than GFP/RV control transduced cells, consistent with the repression of IL-2 promoter transactivation by T-bet that we observed in EL4 cells. The repression of IL-2 by T-bet is consistent with a function for T-bet in driving lineage commitment from a naive precursor cell into a fully differentiated effector cell.

5

Example 8. T-bet activates IFN- γ and represses IL-4 production in developing Th2 cells

The experiments above demonstrate that T-bet can direct unskewed Th cells into the Th1 pathway. The T-bet could force Th cells to direct their genetic program along a 10 Th1 pathway even in the presence of stimuli that would ordinarily drive them into the Th2 pathway was tested. In the experiments in Figure 8, BALB/c CD4+ T cells were activated with anti-CD3 and anti-CD28 in the presence of rIL-4 and antibodies to IFN- γ and IL-12, retroviral infection performed at 36 hours, cells expanded with IL-2, GFP positive cells sorted on day 7 and cytokine production measured by intracellular staining 15 after an additional 4 hours stimulation with PMA plus ionomycin. Transduction with GFP-RV alone resulted in a population that contained 13.4 % IL-4-producing cells and 0.9% IFN- γ producers (Figure 8). As expected, the Thp cells are not yet fully polarized at this time. Introduction of T-bet/GFP/RV produced a substantial shift of Thp into the Th1 pathway as evidenced by the large number of cells (50%) producing IFN- γ and the 20 reduced number of cells producing IL-4 (3.5%), even under conditions (rIL-4 and anti-IL-12) that inhibit Th1 differentiation. Thus, T-bet can overcome the Th2-promoting signals delivered by cytokines to drive developing Th cells into the Th1 pathway.

Example 9. T-bet redirects polarized Th2 cells into the Th1 pathway

25 It has been demonstrated that reversibility of Th1 and Th2 populations is lost after long-term stimulation under polarizing conditions. Reversibility is largely abrogated after one week and is completely lost after 3 weeks (Murphy, E., et al. 1996. *J. Exp. Med.* 183, 901-913.). To determine whether T-bet could redirect the commitment of a pure population of already polarized Th2 cells, CD4+ T cells were cultured as 30 above and retroviral gene transduction performed at day 9 of culture. In Th cells

cultured for 9 days under Th2 polarizing conditions, control GFP/RV-transduced cells are virtually all IL-4 and IL-5 producers (23% and 11%) with barely detectable IFN- γ producer cells (6%) (Figure 9). Thus, as expected, almost complete polarization had occurred. Remarkably, introduction of T-bet into these fully polarized Th2 cells

5 redirected or converted them into polarized Th1 cells as evidenced both by the induction of IFN- γ expression and the loss of IL-4 and IL-5 expression. This conversion occurred in the presence of exogenous IL-4. Fully 77% of T-bet-transduced Th2 cells now produced IFN- γ while the percentage of cells producing IL-4 and IL-5 has been reduced to 13% and 1% respectively. These T-bet-transduced cells are therefore not Th0 cells
10 that produce both IFN- γ and IL-4. Therefore, T-bet has not simply induced IFN- γ expression in Th2 cells but has actually reprogrammed Th2 cells into the opposing Th1 subset.

Example 10. T-bet also redirects polarized Tc2 cells into the Th1 pathway

15 Although most attention has focused on the CD4+ T lymphocyte, it is apparent that cytotoxic CD8+ T cells also may also be divided into IFN- γ -producing (Tc1) and IL-4-producing (Tc2) subsets. The ability of T-bet to redirect fully polarized Tc2 cells into a Tc1 pathway was tested. Purified CD8+ T cells were therefore differentiated in culture under Tc2 polarizing conditions for 9 days to accomplish full differentiation.
20 Figure 10 demonstrates that T-bet transduced Tc2 cells, similar to T-bet transduced CD4 Th2 cells have been reprogrammed to produce IFN- γ (85% versus 15%) and to repress the production of IL-4 and IL-5 (3% versus 34% and 1% versus 45% respectively). Thus, T-bet can convert fully differentiated CD8+ Tc2 cells to Tc1 cells.

25 **Example 11. T-Bet is tyrosine phosphorylated**

To determine whether T-bet is a tyrosine phosphorylated protein, whole cell lysates from AE7 Th1 cells were prepared after incubation for 0, 5, 10, 30 minutes with pervanadate. Lysates were immunoprecipitated with anti-T-bet antiserum, separated by SDS-PAGE (8% gel), transferred to nitrocellulose, and probed with an anti-phosphotyrosine mAB 4G10. Following exposure, blots were stripped and reprobed

with anti-T-bet antisera. As shown in Figure 11, T-bet is clearly a tyrosine phosphorylated protein in T cells.

Example 12. Creation of a dominant negative T-bet molecule

5 Chimeric cDNA molecules were made with the T-bet DNA binding domain (residues 138-327) and the repressor domain of the *Drosophila* protein engrailed. The engrailed protein is a powerful, active repressor of transcription (Taylor, D., 1996. *Genes Dev.* 10, 2732; Li, J., Thurm, H., et al. 1997. *Proc. Natl. Acad. Sci. USA* 94, 10885). The T-bet-*engrailed* construct in vitro using a multimerized T-box consensus 10 site/TK minimal promoter luciferase reporter construct. As shown in Figure 12, T-bet/engrailed specifically and significantly represses the ability of wt T-bet to transactivate a T-box reporter construct at a 5:1 ratio, and does not repress transactivation of an NFAT or NFkB reporter by NFATp and p65 expression constructs respectively.

15

Example 13. Mutations of the T box of the IL-2 promoter decrease IL-2 promoter activity

Recently, the crystal structure of the T-box region of the *Brachury* gene bound to DNA was solved and the amino acid moieties essential for specific DNA contacts or 20 for minor contacts deduced. Examination of the human and murine IL-2 proximal promoter shows that the critical nucleotides for binding a T-box family member are present. Specifically -240 to -220 bp of the murine IL-2 promoter has strong similarity to the consensus T-box site. The consensus T-box site is

AATTCCACCTAGGTGTGAATT. The human IL-2 promoter comprises:

25 gAgTaCACCTAaGTGggcTa. The murine IL-2 promoter comprises: AAacTgcCACCTAaGTGggcTa. The mutated T-box mIL-2 promoter comprises: AAacTgcgtCTAaacaTGggcTa. (DNA contacts are in bold, minor contacts are underlined). Transversional nucleotide substitutions shown by crystal structure to be essential for DNA-protein interactions were made within this putative T-box site in the 30 context of the murine -440 to -40 bp IL-2 promoter. The basal level (open bars) and the PMA (50ng/ml) plus ionomycin (1uM) induced (closed bars) promoter activity in Jurkat

cells (left) or AE7 Th1 clone (right) of IL-2 luciferase reporter constructs is shown (Figure 13).

The role of T-bet is to drive differentiation of Th cells as evidenced by its ability to simultaneously induce the IFN- γ gene and repress the IL-2 gene. The antigen-5 inexperienced Thp cell produces only IL-2. Upon stimulation, IL-2 production declines and is replaced by production of the Th mature effector cytokines. In particular, Th2 cells cease to make IL-2 as they acquire the ability to make IL-4. and the function of T-bet in simultaneously inducing IFN- γ and repressing IL-2 was especially obvious in Th2 cells. The ability of T-bet to simultaneously transactivate the IFN- γ promoter and 10 repress the IL-2 promoter is therefore consistent with a role for T-bet in propelling differentiation of the naïve Thp. T-bet has been shown to transactivate a construct containing only 3 kb of upstream promoter sequence, consistent with the presence of two T-box sites at positions -2300 to -2291 and -1957 to -1948. However, since this region of the promoter is not Th1-specific, it is likely that the T-box site in the third 15 intron is also important and there may well be additional T-box sites elsewhere in the gene.

The T-box domain has recently been co-crystallized with DNA and demonstrates a novel sequence-specific DNA recognition architecture in which the protein contacts DNA in both the major and minor grooves (Müller, C.W. and Herrmann, B.G. 1997. 20 Nature 389, 884-888). The consensus T box binding site as defined by target site selection in vitro is a palindrome 5'-GGGAATTCACACCTAGGTGTGAAATTCCC-3'. Inspection of the IL-2 promoter reveals an excellent T-box site at -240 to -220 just 5' of the NF κ B site to which recombinant T-bet protein binds. The binding of T-bet to the IL-2 promoter explains its isolation in the yeast one hybrid screen where the readout 25 depended simply on binding of T-bet to the T box site in the IL-2 promoter to drive an artificial reporter. Despite the clear repression of IL-2 promoter activity by T-bet, a decrease in IL-2 promoter activity upon mutation of the T-box site has been observed. However, that T-bet can still repress the transactivation of an IL-2 promoter construct in which that T-box site has been mutated. This suggests either the presence of another T- 30 box site in the IL-2 promoter, or interference with another positively acting factor that

binds close by. A good candidate for this factor is an activity described by Rothenberg and colleagues that binds to a site TGGGCC just adjacent to the T-box site (Chen, D. and Rothenberg, E.V. 1994. *J. Exp. Med.* **179**, 931-942).

In addition, T-bet represses the Th2 program in Thp and Th2 cells. This is

5 unlikely to be the direct result of an imbalance between IFN- γ and IL-4 in favor of the former. The effect of T-bet in repressing the Th2 program while simultaneously enhancing the Th1 program is reminiscent of GATA-3 and c-Maf, both of which indirectly repress IFN- γ expression, the former through influencing expression of the IL-12 receptor β 2 chain (Ho, I-C., et al. 1998. *J. Exp. Med.* **188**:1859-1866.; Ouyang, 10 W., et al. 1998. Inhibition of Th1 developmental mediated by GATA-3 through an IL-4 independent mechanism. *Immunity* **9**:745-755). However, unlike GATA-3 and c-Maf, T-bet can actually convert fully polarized effector Th cells into the opposing pathway.

Equivalents

15 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

1. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a T-bet protein.

5

2. The nucleic acid molecule of claim 1, which comprises the nucleotide sequence shown in SEQ ID NO:1.

3. The nucleic acid molecule of claim 2, which comprises the nucleotide sequence of SEQ ID NO: 3.

4. The nucleic acid molecule of claim 1, which has at least 70% nucleotide identity with at least about 700 contiguous nucleotides of SEQ ID NO:1.

15 5. The nucleic acid molecule of claim 1, which has at least 70% nucleotide identity with at least about 500 contiguous nucleotides of SEQ ID NO:3.

6. The nucleic acid molecule of claim 1, which has at least 90% nucleotide identity with at least about 700 contiguous nucleotides of SEQ ID NO:1.

20

7. The nucleic acid molecule of claim 1, which has at least 90% nucleotide identity with at least about 500 contiguous nucleotides of SEQ ID NO:3.

25

8. A vector comprising the nucleic acid molecule of claim 1.

9. The vector of claim 8, which is an expression vector.

10. A host cell containing the vector of claim 9.

30

11. A method for producing a T-bet protein comprising culturing the host cell of claim 10 in a suitable medium until a T-bet protein is produced.

12. The method of claim 11, further comprising isolating the T-bet protein from the medium or the host cell.

5 13. An isolated T-bet protein.

14. The protein of claim 12, which comprises the amino acid sequence encoded by the nucleic acid sequence of SEQ ID NO:1.

10 15. The protein of claim 12, which comprises the amino acid sequence encoded by the nucleic acid sequence of SEQ ID NO:3.

15 16. The protein of claim 12, which comprises the amino acid sequence of SEQ ID NO: 2.

17. The protein of claim 12, which comprises the amino acid sequence of SEQ ID NO: 4.

18. The protein of claim 12, which has at least 70% amino acid identity with 20 the protein shown in SEQ ID NO:2.

19. The protein of claim 12, which has at least 70% amino acid identity with the protein shown in SEQ ID NO:4.

25 20. The protein of claim 12, which has at least 90% amino acid identity with the protein shown in SEQ ID NO:2 and having the ability to bind to DNA.

21. A fusion protein comprising a T-bet protein operatively linked to a 30 polypeptide other than T-bet.

22. Antibodies that specifically bind T-bet protein.

23. The antibodies of claim 21, which are polyclonal antibodies.

24. The antibodies of claim 21, which are monoclonal antibodies.

5 25. The antibodies of claim 21, which are coupled to a detectable substance.

26. A nonhuman transgenic animal that contains cells carrying a transgene 10 encoding a T-bet protein.

10 27. A method for detecting the presence of T-bet in a biological sample comprising contacting the biological sample with an agent capable of detecting an indicator of T-bet activity such that the presence of T-bet is detected in the biological sample.

15 28. A method for modulating T-bet activity in a cell comprising contacting the cell with an agent that modulates T-bet activity such that T-bet activity in the cell is modulated.

20 29. A method for identifying a compound that modulates the activity of a T-bet protein, comprising

providing an indicator composition that comprises a T-bet protein;

contacting the indicator composition with a test compound; and

determining the effect of the test compound on the activity of the T-bet protein in 25 the indicator composition to thereby identify a compound that modulates the activity of a T-bet protein.

- 88 -

30. The method of claim 27, wherein:

the indicator composition comprises a T-bet protein and a DNA molecule to which the T-bet protein binds; and

the effect of the test compound on the activity of the T-bet protein is determined

5 by evaluating the binding of the T-bet protein to the DNA molecule in the presence and absence of the test compound.

31. The method of claim 27, wherein:

the indicator composition is a cell comprising a T-bet protein and a reporter gene

10 responsive to the T-bet protein; and

the effect of the test compound on the activity of the T-bet protein is determined by evaluating the expression of the reporter gene in the presence and absence of the test compound.

15 32. The method of claim 27, further comprising determining the effect of the test compound on an immune response to thereby identify a compound that modulates an immune response.

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	10	20	30	40	50	60
HUMAN	MGIVEPGCGDMLTGTEPMGSDEGRAPGADPQHRYFYPEPEGAQDADERRGGGSLGSPY					
MOUSE	MGIVEPGCGDMLTGTEPMPSDEGRGPGADQHRRFFYYPEPEGAQDPTDRAGSSLGTPY					
	10	20	30	40	50	

CONS	MGIVEPGCGDMLTGTEPMPSDEGRPGADQHRYFYPEPEGAQD	RR	G	SLG	PY	G
------	--	----	---	-----	----	---

	70	80	90	100	110	120
HUMAN	GALVPAPPSSRFLGAYAYPPRPQAAGFPAGESFPPPPADAEGYQPGEGYAAPDPRAGLY					
MOUSE	GALVPAAPGRFLGSFAYPPRAQVAGFPGPGEFPAGAEGYPPVVDYPAPDPRAGLY					
	60	70	80	90	100	110

CONS	GALVPA P RFLG AYPPR Q AGFPGE FPPPA AEGY P GY APDPRAGLY					
------	--	--	--	--	--	--

	130	140	150	160	170	180
HUMAN	PREDYALPAGLEVSGKLRLVALNHLWSKFQNQHTEMIIITKQGRRMFPFLSFTVAGLE					
MOUSE	PREDYALPAGLEVSGKLRLVALNHLLWSKFQNQHTEMIIITKQGRRMFPFLSFTVAGLE					
	120	130	140	150	160	170

CONS	PREDYALPAGLEVSGKLRLVALNHLLWSKFQNQHTEMIIITKQGRRMFPFLSFTVAGLE					
------	---	--	--	--	--	--

	190	200	210	220	230	240
HUMAN	SHYRMFVDVVLVDQHHWRYSQSGKWWQCGKAEGSMGPGNRLYVHPDSPNTGAHWMRQEVS					
MOUSE	SHYRMFVDVVLVDQHHWRYSQSGKWWQCGKAEGSMGPGNRLYVHPDSPNTGAHWMRQEVS					
	180	190	200	210	220	230

CONS	SHYRMFVDVVLVDQHHWRYSQSGKWWQCGKAEGSMGPGNRLYVHPDSPNTGAHWMRQEVS					
------	--	--	--	--	--	--

	250	260	270	280	290	300
HUMAN	KLKLTNNGASNNVTQMIQLQSLHKYQPRLHIVEVNDGEPEAACNASNTHIFTFQETQFI					
MOUSE	KLKLTNNGASNNVTQMIQLQSLHKYQPRLHIVEVNDGEPEAACSASNTHVFTFQETQFI					
	240	250	260	270	280	290

CONS	KLKLTNNGASNNVTQMIQLQSLHKYQPRLHIVEVNDGEPEAAC ASNTH FTFQETQFI					
------	---	--	--	--	--	--

Fig. 1A

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	310	320	330	340	350	360
HUMAN	AVTAYQNAEITQLKIDNNPFAKGFRNFESMYTSVDT	SIPSPPGPNCQFLGGDHYSPLLP				
MOUSE	AVTAYQNAEITQLKIDNNPFAKGFRNFESMYASVDT	SVFSPPGPNCQLLGGDPFSPLLS				
	300	310	320	330	340	350

CONS AVTAYQNAEITQLKIDNNPFAKGFRNFESMY SVDTS PSPPGPNCQ LGGD FSPLL

	370	380	390	400	410	420
HUMAN	NQYPVPSRFYPDLPQAKDVPQAYWLGA	PRDHYSYEAEFRAVSMKP	FLPSAPGPTMSYY			
MOUSE	NQYPVPSRFYPDLPQPKDMISQPYWLGT	PREHYSYEAEFRAVSMKP	TLPLPSAPGPTVYY			
	360	370	380	390	400	410

CONS NQYPVPSRFYPDLPQ KD Q YWLG PR HSYEAEFRAVSMKP LPSAPGPT YY

	430	440	450	460	470	480
HUMAN	RGQEVLAGAGWPVAPQYPKMKPASWFRPM	RMLPMEPGPGGSEGRGPEDQGPP	PLVWTEI			
MOUSE	RGQDVLAGAGWPVAPQYPKMS	PAGWFRPMRMLPMDPLGGS	SEEQG---SSPSLWPEV			
	420	430	440	450	460	470

CONS RGQ VLAPGAGWPVAPQYPKMK PA WFRPMRMLP PG G SE G P W E

	490	500	510	520	530	
HUMAN	APIRPESSDSGLGEGDSKRRRVSPY	PSSGDSSSPAGAPSFDKEAEGQFYNTFPN				
MOUSE	TSLQEPSPDSDLGEGDTKRRRIS	PSPYSPSSGDSSSPAGAPSFDKE	TEGQFYNYFPN			
	480	490	500	510	520	530
CONS	PE SDSGLGEGL KRRR SPYSPSSGDSSSPAGAPSFDKE	<u>EGQFYNYFPN</u>				

Fig. 1A (continued)

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	10	20	30	40	50	60
HUMAN	ATGGGCATCGTGGAGCCGGTTGCGGAGACATGCTGACGGGACCCGAGCCGATGCCGGG					
MOUSE	ATGGGCATCGTGGAGCCGGCTGCGGAGACATGCTGACCGGACCCGAGCCGATGCC	---	G			
	10	20	30	40	50	
	70	80	90	100	110	120
HUMAN	AGCGACGAGGGCCGGGCGCTGGCGCCGACCCGACGCACCGCTACTTCTACCCGGAGCCG					
MOUSE	AGT GACGAGGGCCGGGCCCCGGAGCGGACACAGCATCGTTCTTATACCCGAGCCG					
	60	70	80	90	100	110
	130	140	150	160	170	180
HUMAN	GGCGCGCAGGACGCGGACGAGCGTCGCGGGGGCGGCAGCCTGGGTCTCCCTACCCGGG					
MOUSE	GGCGCACAGGACCCGACCGATCGCGCGCAGGTAGCAGCCTGGGAGCCCTACTCTGGG					
	120	130	140	150	160	170
	190	200	210	220	230	240
HUMAN	GGGCCTTGGTGCCCGCCCGCGAGCCGCTTCTGGAGCCTACGCCCTACCCGGCGCGA					
MOUSE	GGGCCTTGGTGCCTGCCCGCCGGGTCGCTTGGATCTTCGCCCTACCCGGGGGG					
	180	190	200	210	220	230
	250	260	270	280	290	300
HUMAN	CCCCAGGGCCGGCTTCCCGGGCGGGAGTCCTCCGCCGCCGGAGCGGAG					
MOUSE	GCTCAGGTGGCTGGCTTCCCGGGCTGGCAGTTCTCCGCCGCCGGGGTGGGGAG					
	240	250	260	270	280	290
	310	320	330	340	350	360
HUMAN	GGCTACCAAGCCGGCGAGGGCTACCCGCCCGGACCCGCCGGGGCTACCCGGG					
MOUSE	GGCTACCCGCCCTGGATGGCTACCCCTGCCCTGACCCGCCGGGGCTACCCAGGG					
	300	310	320	330	340	350
	370	380	390	400	410	420
HUMAN	CCCGCTGAGGACTACGCCCTACCCGGGGACTGGAGGTGCGGGAAACTGAGGGTCGCG					
MOUSE	CCCGCGAGGACTACGCAATTGCCCGGGGTTGGAGGTGCTGGGAAGCTGAGAGTCGCG					
	360	370	380	390	400	410

Fig. 1B

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	430	440	450	460	470	480
HUMAN	CTCAACAACCACCTGTTGCTCAAGTTAACATCAGCACCAAGACAGAGATGATCATCAC					
MOUSE	CTCAGCAACCACCTGTTGCTCAAGTTAACCCAGCACAGACAGAGATGATCATCCT					
	420	430	440	450	460	470
	490	500	510	520	530	540
HUMAN	AAGCAGGGACGGCGATGTTCCATTCTGTCAATTACTGTTGGCGGGCTGGAGGCCACC					
MOUSE	AAGCAAGGACGGGAATGTTCCATTCTGTCTTACCGTGGCGGGCTGGAGGCCACA					
	480	490	500	510	520	530
	550	560	570	580	590	600
HUMAN	AGCCACTACAGGATGTTGTTGGACGTCGGCTTGGTGGACAGCACCCTGGGGTACAG					
MOUSE	AGCCATTACAGGATGTTGTTGGATGTTGGACAGCACCCTGGGGTACAG					
	540	550	560	570	580	590
	610	620	630	640	650	660
HUMAN	AGCGGCAAGTGGGTGCACTGGAAAGGCCAGGGCAGCATGCCAGGAACCGCCTGTAC					
MOUSE	AGCGGCAAGTGGGTGCACTGGAAAGGCAGAGGCAGCATGCCAGGAACCGCTTATAT					
	600	610	620	630	640	650
	670	680	690	700	710	720
HUMAN	GTCCACCCGACTCCCCAACACAGGAGCGCACTGGATGCCAGGAAGTTCAATTGGG					
MOUSE	GTCCACCCGACTCCCCAACACCGGAGGCCACTGGATGCCAGGAAGTTCAATTGGG					
	660	670	680	690	700	710
	730	740	750	760	770	780
HUMAN	AAACTAAAGCTCACAAACACAAGGGGGCTCCAACAAATGTGACCCAGATGATTGCTC					
MOUSE	AAGCTAAAGCTCACAAACACAAGGGGGCTCCAACAAATGTGACCCAGATGATCGTCTG					
	720	730	740	750	760	770
	790	800	810	820	830	840
HUMAN	CAGTCCCTCCATAAGTACAGCCCCGGCTGCATATCGTTGGGTGAACGACGGAGAGCCA					
MOUSE	CAGTCTCTCCACAAAGTACAGCCCCGGCTGCACATCGTGGAGGTGAATGATGGAGAGCCA					
	780	790	800	810	820	830

Fig. 1B (continued)

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HUMAN	850	860	870	880	890	900
	GAGGCAGCTGCAACGCTTCCAACACGCATATCTTTACTTCCAAAGAAACCCAGTTATT					
MOUSE	840	850	860	870	880	890
	GAGGCTGCCTGCACTGCTTAACACACACGCTTTACTTCCAAAGAGACCCAGTTATT					
HUMAN	910	920	930	940	950	960
	GCCGTGACTGCCTACCAGAAATGCCGAGATTAACGTGAAAATTGATAATAACCCCTT					
MOUSE	900	910	920	930	940	950
	GCAGTGACTGCCTACCAGAAACGCCGAGATCACTCAGCTGAAAATGACAACACCCCTT					
HUMAN	970	980	990	1000	1010	1020
	GCCAAAGGATTCCGGGAGAACCTTGAGTCCATGTACACATCTGTTGACACCCAGCATCCCC					
MOUSE	960	970	980	990	1000	1010
	GCCAAAGGATTCCGGGAGAACCTTGAGTCCATGTACGCACTGTTGATAACGAGTGTCCCC					
HUMAN	1030	1040	1050	1060	1070	1080
	TCCCCGCCTGGACCCAACGTCAATTCTGGGGGAGATCACTACTCTCTCTCTACCC					
MOUSE	1020	1030	1040	1050	1060	1070
	TCGCCACCTGGACCCAACGTCAACTGCTGGCTGGGGGAGACCCCTCTCACCTCTATCC					
HUMAN	1090	1100	1110	1120	1130	1140
	AACCAGTATCCCTGTTCCCAGCCGTTCTACCCCGACCTTCTGGCCAGGCCAGGATGTG					
MOUSE	1080	1090	1100	1110	1120	1130
	AACCAGTATCCCTGTTCCCAGCCGTTCTACCCCGACCTTCCAGGCCAGGATATG					
HUMAN	1150	1160	1170	1180	1190	1200
	GTTCCCCAGGCTTACTGGCTGGGGCCCCCGGGACAGCTATGAGGCTGAGTTTGA					
MOUSE	1140	1150	1160	1170	1180	1190
	ATCTCACCCCTTACTGGCTGGGGACACCTCGGGAACACAGTTATGAAGCCGAGTTCCG					
HUMAN	1210	1220	1230	1240	1250	1260
	GCAGTCAGCATGAAGCTGCATTCTGGCCCTCTGCCCTGGGCCACCATGTCTACTAC					
MOUSE	1200	1210	1220	1230	1240	1250
	GCTGTCAGCATGAAGCCCACACTCTACCCCTCTGCCCTGGGCCACCATGTCTACTAC					

Fig. 1B (continued)

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HUMAN	CGAGGCCAGGAGGTCTGGCACCTGGAGCTGGCTGGCCCTGGCACCCAGTACCCCTCC	1270	1280	1290	1300	1310	1320
MOUSE	CGGGGCCAAGACGTCTGGCGCTGGAGCTGGTTGGCCCTCAATACCCGCC	1260	1270	1280	1290	1300	1310
HUMAN	AAGATGGGCCGGCAGCTGGTCCCGCCCTATGCGACTCTGCCATGGAACCCGGCC	1330	1340	1350	1360	1370	1380
MOUSE	AAGATGAGGCCAGCTGGCTGGTCCGGCCATGCGAACCTGCCATGGACC	1320	1330	1340	1350	1360	1370
HUMAN	GGAGGCTCAGAGGGACGGGACCAAGAGGCCAGGGTCCCCCTGGGTGACTGAGATT	1390	1400	1410	1420	1430	1440
MOUSE	GGATCCTCAGAGAACAGGGCTCCT-----CCCCCTCGCTGTGGCCTGAGGTC	1380	1390	1400	1410	1420	
HUMAN	GCCCCCATCGCCCGGAATCCAGTGATTCAAGACTGGGCGAAGGAGACTCTAAGAGGAGG	1450	1460	1470	1480	1490	1500
MOUSE	ACCTCCCTCAAGCGGAGCCCAGGGACTCAAGGACTAGGGCAAGGAGACACTAAGAGGAGG	1430	1440	1450	1460	1470	1480
HUMAN	CGCGTGTCCCCCTATCCTTCCAGTGGTGACAGCTCTCCCTGCTGGGGCCCTCTCCT	1510	1520	1530	1540	1550	1560
MOUSE	AGGATATCCCCCTATCCTTCCAGTGGTGACAGCTCTCCCTGCTGGGGCCCTCTCCT	1490	1500	1510	1520	1530	1540
HUMAN	TTTGATAAGGAAGCTGAAGGACAGTTTATAACTATTTTCCAACTGAA	1570	1580	1590	1600		
MOUSE	TTTGATAAGGAACCGAAGGCCAGTTTATAATTATTTCCAACTGAA	1550	1560	1570	1580	1590	

Fig. 1B (continued)

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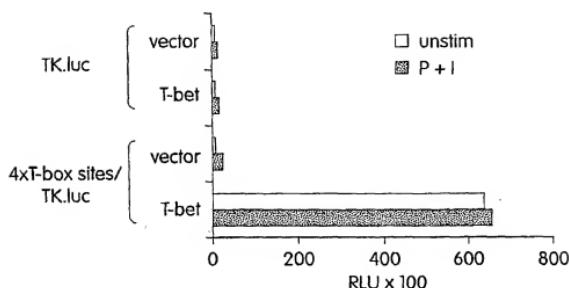


Fig. 2A

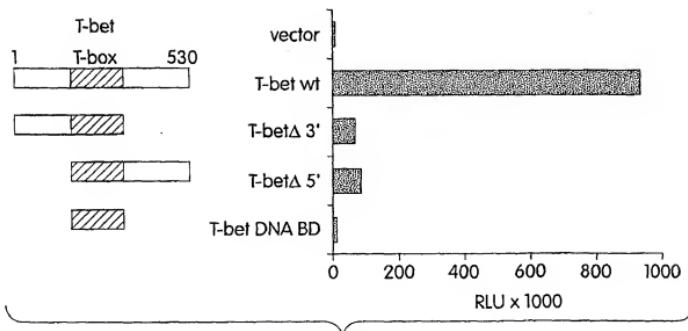


Fig. 2B

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Fig. 3A



Fig. 3B

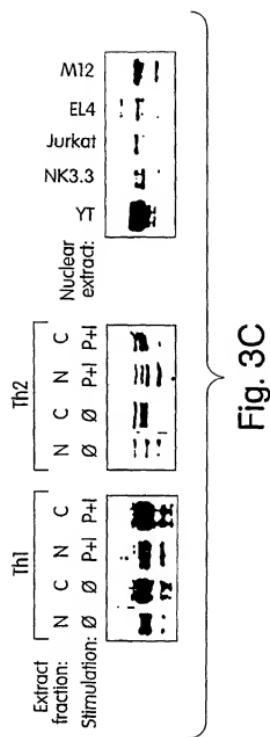


Fig. 3C

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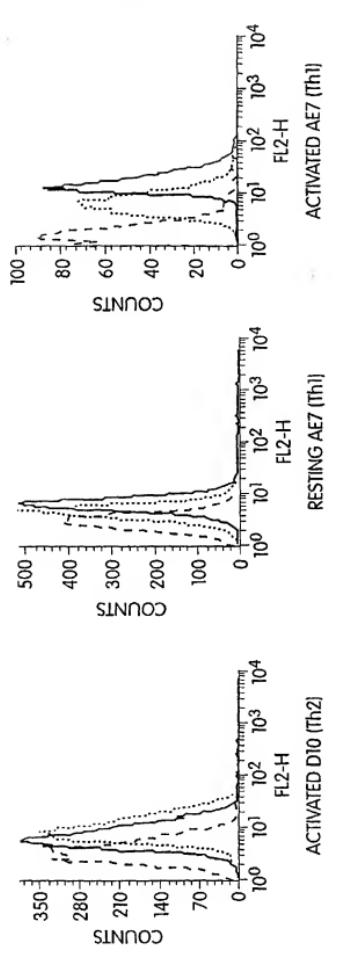


Fig. 3D

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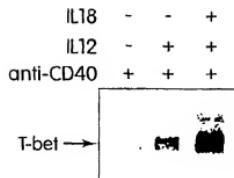


Fig. 4A

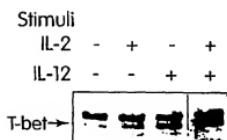


Fig. 4B

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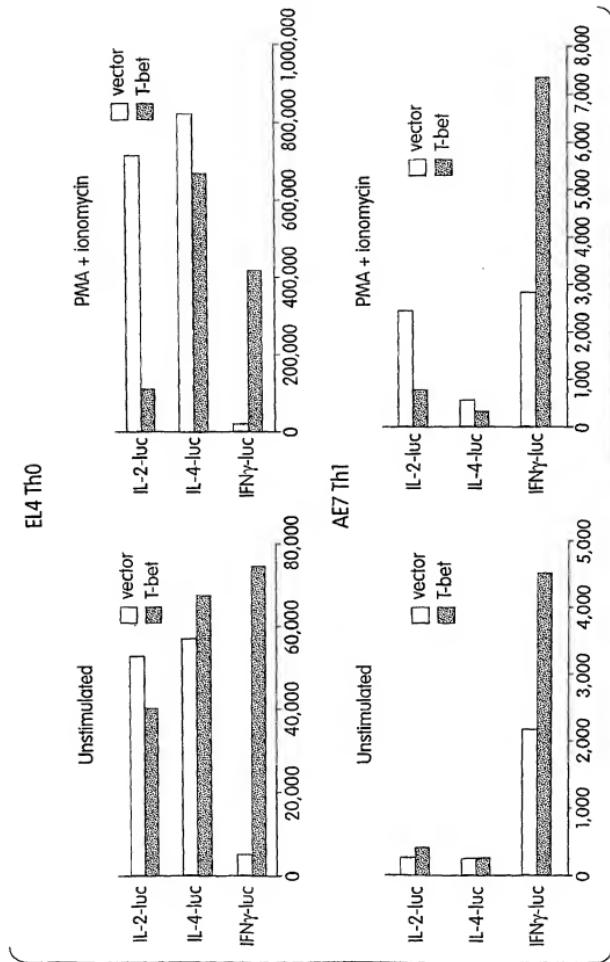


Fig. 5

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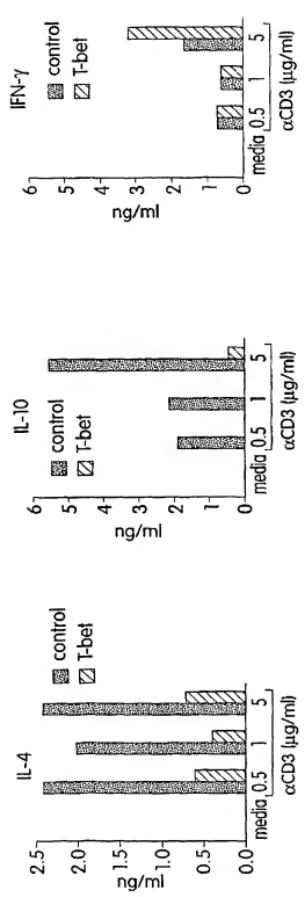


Fig. 6

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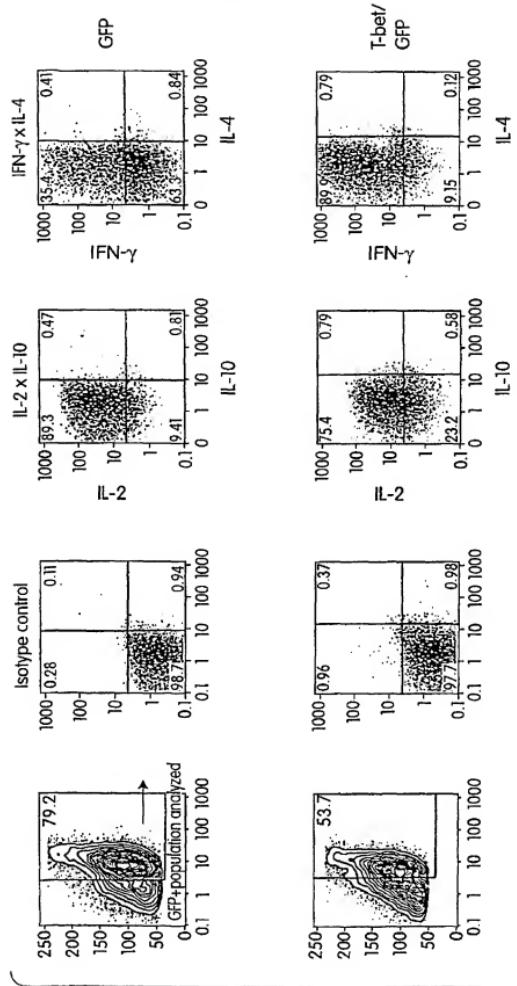


Fig. 7

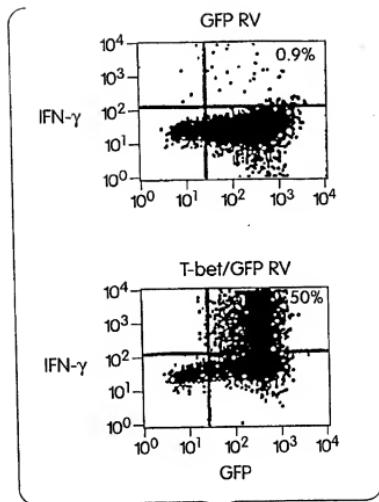
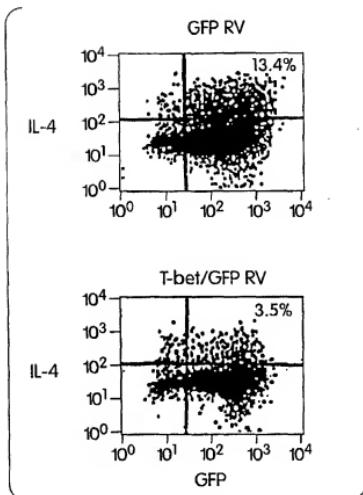


Fig 8A



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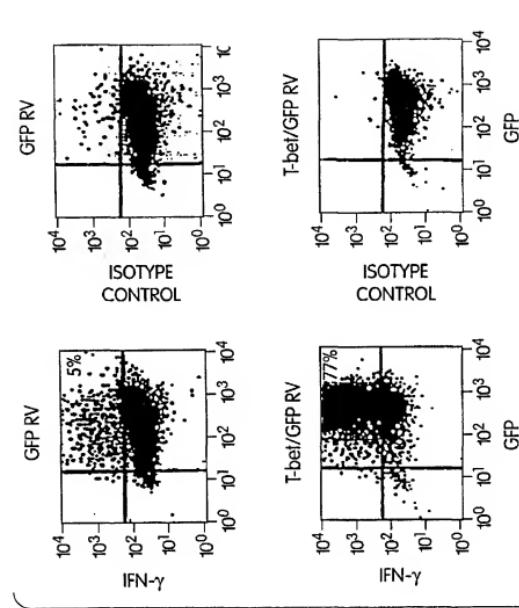
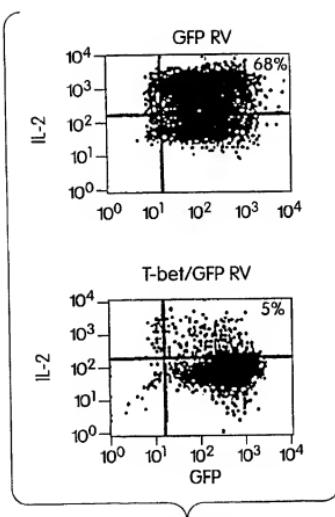


Fig 9A

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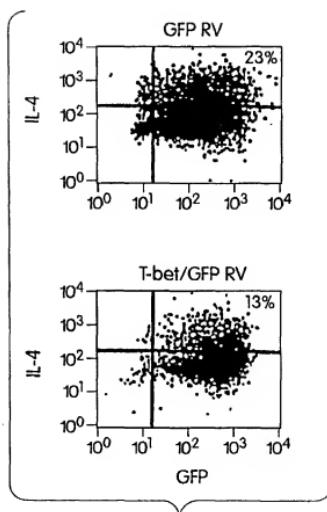


Fig 9C

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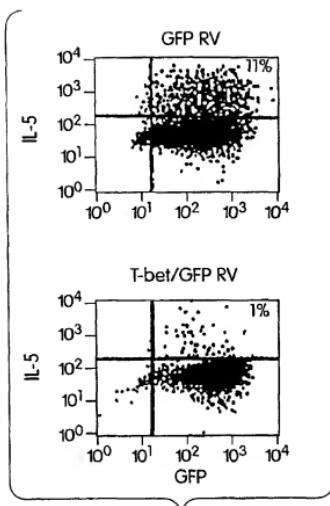


Fig 9D

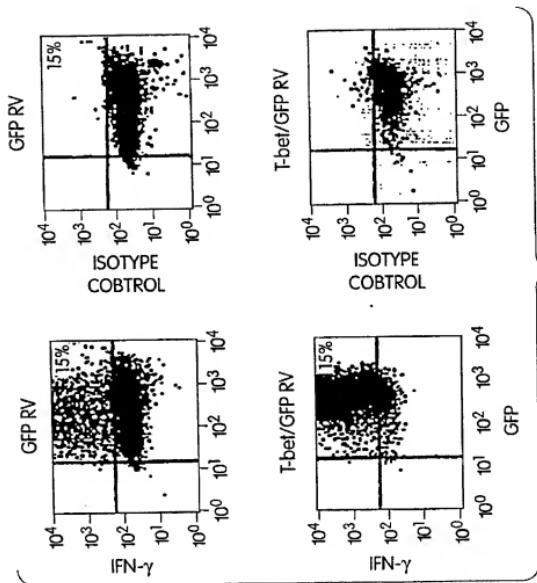


Fig 10A

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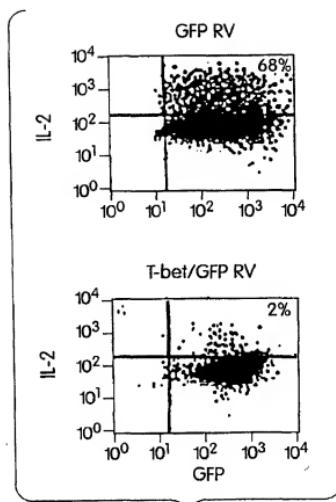


Fig. 10B

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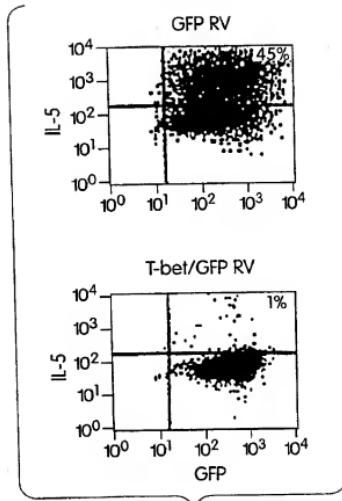


Fig. 10C

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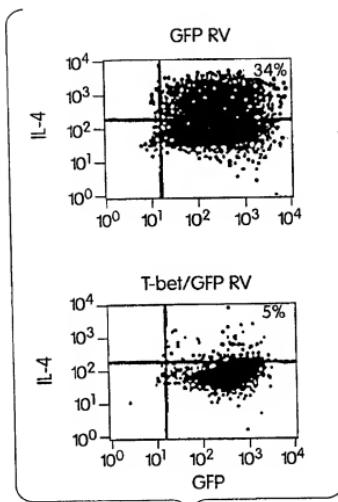
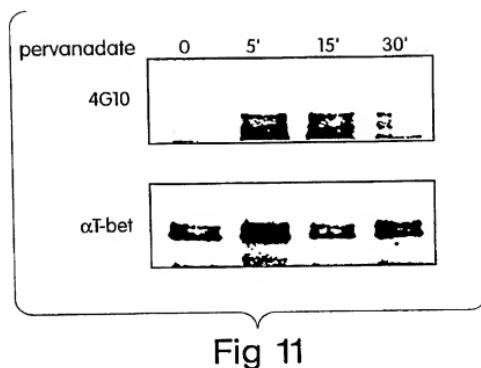


Fig. 10D

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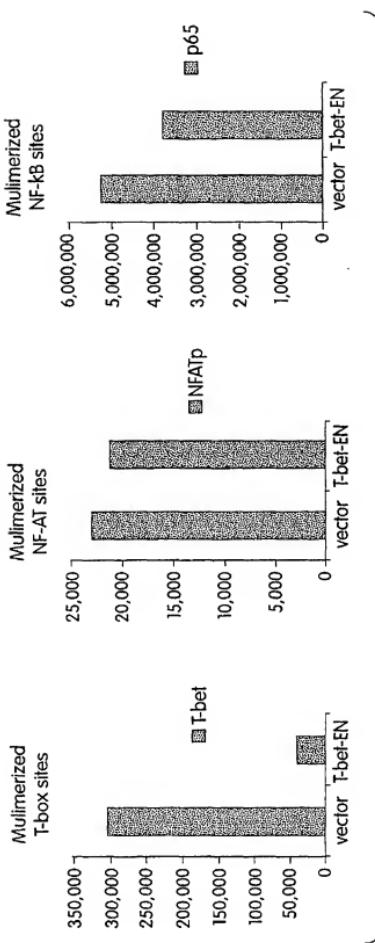


Fig. 12

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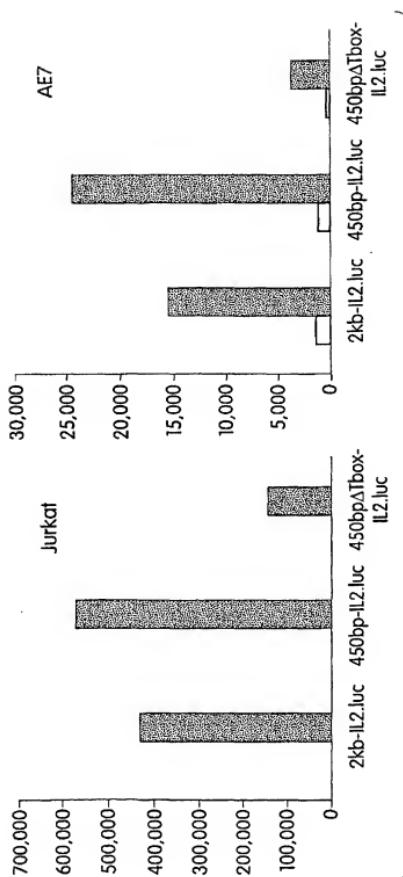


Fig. 13

- 1 -

SEQUENCE LISTING

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 00/15345

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C12N15/12 C12N5/10 C07K14/47 C07K16/18 G01N33/50
 C12Q1/68 A01K67/027 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 C12N C07K G01N C12Q A01K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, STRAND, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	SZABO S.J. ET AL.: "A novel transcription factor, T-bet, directs Th1 lineage commitment." CELL, vol. 100, March 2000 (2000-03), pages 655-669, XP002148616 the whole document ----- DATABASE EMBL SEQUENCES 'Online!' Accession No. AF093098, 16 October 1999 (1999-10-16) YANG S.: "Cloning and characterization of a new member of T-box gene Family (H. sapiens TBLYM)." XP002148618 TBLYM is identical to T-bet ----- -/-	1-32
P, X	-----	1-32

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may allow double on priority claim(s) or which is cited to establish the priority date of another document or special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/15345

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BULFONE A ET AL: "T-brain-1: a homolog of Brachury whose expression defines molecularly distinct domains within the cerebral cortex" NEURON, CAMBRIDGE, MA, US, vol. 15, no. 1, July 1995 (1995-07), pages 63-78, XP000864769 the whole document -----	1-32
A	SMITH J: "T-box genes: what they do and how they do it" TRENDS IN GENETICS, NL, ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, vol. 15, no. 4, April 1999 (1999-04), pages 154-158, XP004162854 ISSN: 0168-9525 the whole document -----	1-32
A	NOBLE A. ET AL.: "Regulation of Tc1/Tc2 and Th1/Th2 subset commitment by altered peptides and TcR-mediated signalling events." FASEB J., vol. 13, no. suppl., 15 March 1999 (1999-03-15), page A939 XP002148617 the whole document -----	1-32